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THE CARBOHYDRATE METABOLISM OF BRAIN

I. THE DETERMINATION OF GLYCOGEN IN NERVE TISSUE*

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The various modifications of the Pflüger (1904) procedure commonly used for determining glycogen in tissues yield erroneous results when applied to brain or nerve, owing to the presence of one or more compounds which on acid hydrolysis yield reducing substances other than glucose. Winterstein and Hirschberg (1925) pointed out that cerebrosides as well as glycogen are found in the precipitate formed by alcohol, and that the subsequent hydrolysis by acid liberates sufficient galactose to introduce gross errors. The values reported by various investigators for mammalian brain vary from 0 to 269 mg. per 100 gm. This wide variation appears to be due in part to incomplete removal of cerebrosides, to the use of methods for estimating glucose which include certain non-sugar reducing substances, to loss of glycogen during the attempted separation of cerebrosides, and to various degrees of postmortem autolysis.

The procedure described below was designed to avoid these errors. The brain is frozen *in situ* with liquid air and brought into solution with hot alcoholic potassium hydroxide. The cerebrosides are separated by solution in a methyl alcohol-chloroform mixture. After hydrolysis of the remaining insoluble material the sugar is estimated by means of the Shaffer-Somogyi (1933) procedure and corrected for reducing substances not destroyed by yeast fermentation.

* Since most of the evidence which points to the presence of glycogen in brain is indirect, we have attempted to isolate the material in pure form as a more satisfactory proof of its existence. A report on the method of isolation and the properties of the compound will be published shortly.

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Reagents—

Alcoholic potassium hydroxide (20 per cent KOH in 60 per cent alcohol), prepared by mixing 1 volume of 60 per cent KOH with 2 volumes of 90 per cent alcohol. When this becomes brown owing to formation of aldehyde resins, it should be discarded.

Methyl alcohol-chloroform mixture (80 volumes of methyl alcohol and 20 of chloroform).

The copper-iodometric reagents of Shaffer and Somogyi (1933) for sugar determination. The reagent containing 1 gm. of KI and 20 cc. of N iodate per liter is best suited to the quantities of carbohydrate determined in brain. We titrate with 0.01 N thiosulfate, using a microburette graduated in 0.01 cc.

Phenol red indicator (0.1 per cent aqueous solution).

Phosphate buffer, pH 6.6, with indicator. Dissolve 13.6 gm. of KH_2PO_4 in water, add 35.6 cc. of N NaOH, and dilute to 100 cc. Add 50 cc. of 0.1 per cent aqueous solution of brom-cresol purple.

Suspension of washed yeast (25 per cent), prepared as described by Raymond and Blanco (1928).

Tungstic acid solution, prepared daily by mixing equal parts of 10 per cent sodium tungstate and $\frac{2}{3}$ N sulfuric acid.

Normal HCl.

5 N NaOH.

Method

Transfer 10 cc. of alcoholic potassium hydroxide to a Pyrex test-tube (150 × 18 mm.) graduated at 12.5 cc., stopper, and weigh. Add 1.5 to 3.5 gm. of brain, prepared with precautions to avoid postmortem change, as described elsewhere (Kerr, 1935). The frozen powdered brain should be transferred to the alcoholic potassium hydroxide through a small Pyrex funnel with a wide (15 mm.) stem, chilled in liquid air, in order to avoid loss of the specimen in the upper portions of the tube. Rotate the tube at once to mix the specimen with the potassium hydroxide and reweigh. Remove the stopper and immerse the tube in a bath at 80–85° for 10 minutes, mixing occasionally. Just before removing from the bath raise the temperature until the mixture begins to boil, then allow it to cool spontaneously. Centrifuge for 30 minutes at about 2800 R.P.M., then decant and discard the supernatant fluid. Now separate the cerebrosides by washing the precipitate

three times with hot methyl alcohol-chloroform¹ mixture. The washing is best accomplished by adding 10 cc. of the solvent and immersing the tube in hot water until the mixture boils. After the contents of the tube have cooled, centrifuge sharply for 10 minutes and decant the fluid. After the third washing immerse the tube in boiling water to drive off the remaining traces of the solvent.

To the dried precipitate add 10 cc. of *N* HCl and hydrolyze in a boiling water bath for 2 hours.¹ To avoid excessive evaporation, insert in the mouth of the tube a loosely fitting glass stopper (flat topped), or a sealed glass bulb such as is suggested by Shaffer and Somogyi (1933). Add 1 drop of phenol red, neutralize with 5 *N* NaOH, then acidify with *N* HCl, taking care that not more than 1 drop in excess of acid is added. Dilute to 12.5 cc., mix, and pour through a dry filter.

Determine the "apparent" sugar in 5 cc. of the filtrate (equivalent to 0.4 of the original brain specimen) by the method of Shaffer and Somogyi (1933). The "residual" reducing substances are determined by the method of Raymond and Blanco (1928). Measure 5 cc. of the filtered hydrolysate into a 15 cc. centrifuge tube, and add 3 drops of the phosphate buffer-indicator mixture. If the pH does not fall within the range 6.0 to 6.6,² adjust with *N* NaOH, then add 2 cc. of 25 per cent yeast suspension, mix, allow to stand 4 minutes, then add 1 cc. (less 3 drops) of the tungstic acid solution. The total volume is now 8.0 cc. Again mix and centrifuge. Pipette off 5 cc. of the supernatant fluid (equivalent to exactly one-fourth of the original brain specimen) and determine the reducing substances by the Shaffer-Somogyi procedure.

The difference between the values for "apparent" and "residual" sugar, calculated on the basis of 100 gm. of brain, represents the

¹ Sahyun and Alsberg (1931) found that the hydrolysis of glycogen in *N* HCl or H₂SO₄ is complete in 90 minutes.

² The 3 drops of buffer added contain the equivalent of 1 drop of 0.1 per cent brom-cresol purple. 1 drop of phenol red (0.1 per cent) was previously added during neutralization. This mixture of equal parts of the two indicators permits the adjustment of acidity to the desired pH (6.6) with ease, by comparing with a tube containing 5 cc. of water to which are added 3 drops of the buffer-indicator mixture and a drop of phenol red. As long as the color is neither yellow nor purple, the acidity is suitable for fermentation by yeast.

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true glycogen. This should be multiplied by the usual factor 0.927 to correct for incomplete hydrolysis and change in molecular weight (Nerking, 1901), thus converting glucose found to anhydrous glycogen.

DISCUSSION

The rapidity with which lactic acid formation proceeds in brain after death or excision of the tissue (Avery, Kerr, and Ghantus, 1935), necessitates freezing *in situ* if resting values for carbohydrates are desired.

In the Pflüger procedure for glycogen 3 hours heating with aqueous KOH was recommended for the sake of destroying certain interfering reducing substances. Prolonged heating, however, does not accomplish this purpose when one is working with brain; hence the period of digestion may be shortened to the time necessary for complete disintegration of the tissue, since correction is made for the non-glucose reducing substances by means of yeast fermentation. With brain the time required for dissolving the tissue is considerably shortened by the use of alcoholic potassium hydroxide in place of the customary aqueous solution. The results are identical whether the specimen is heated for 3 hours in KOH or as described above.

The various methods used by other workers for removal of cerebroside have proved unsatisfactory in our hands. Winterstein and Hirschberg (1925) used two procedures, but finally adopted the method of extracting the glycogen from the alcohol-insoluble material with boiling water, and separating the cerebroside by centrifugation. We were unable to make the separation at all satisfactory either by centrifugation or filtration, owing to the colloidal nature of the extracts, and found high values for reducing substances other than glucose after hydrolysis of such extracts.

Winterstein and Hirschberg (1925) stated that the removal of cerebroside by means of solvents (particularly chloroform) was unsatisfactory. We have been unable to detect any loss of glycogen after repeated washings with the methyl alcohol-chloroform mixture. The cerebroside is readily dissolved by the hot solvent, but a considerable amount may reprecipitate if the washings are carried out in a cold room or if long periods are

allowed to elapse before centrifuging. Even if the removal of cerebrosides is incomplete after three washings, the determination of non-fermentable reducing substance corrects for the error. This amounts usually to less than 5 mg. per 100 gm., but occasionally may be as great as 40 mg., owing possibly to reprecipitation from the solvent.

Other solvents have been used with partial success. Extraction of the alcohol-insoluble material with boiling ethyl alcohol followed by filtration through a small steam-jacketed funnel permitted recovery of about 90 per cent of added glycogen, the loss being due to adsorption of glycogen on either filter paper, pulp or asbestos. The presence of a gummy substance insoluble in the hot alcohol also interfered with subsequent extraction of the glycogen from the paper or asbestos.

The Shaffer-Somogyi (1933) procedure for measuring the reducing substances in the glycogen hydrolysate was chosen in preference to the Hagedorn-Jensen, because the latter is much more sensitive to reducing substances other than glucose. The "apparent" sugar in the hydrolysate as measured by the Hagedorn-Jensen method is as much as 50 per cent higher than that found when the Shaffer-Somogyi method is used. On correction for "residual" reducing substances, however, this discrepancy disappears.

The fact that a much larger amount of non-fermentable reducing substance is measured by the Hagedorn-Jensen than by the Shaffer-Somogyi procedure is evidence that more than one "residual" reducing substance is present in the glycogen hydrolysate. Four extractions of the glycogen precipitate with methyl alcohol and chloroform bring the residual sugar to no lower level than three, but the amount remaining may nevertheless be due to incomplete removal of cerebrosides. The non-fermentable reducing substance is liberated by the acid hydrolysis, since an aqueous extract of the washed glycogen precipitate before hydrolysis contains no reducing substance, as measured by the Shaffer-Somogyi method. The precipitation of zinc hydroxide in the glycogen hydrolysate does not remove the non-fermentable reducing substance.

Glycogen added to brain is satisfactorily recovered by the procedure described. The recovery varied from 86.5 to 99.4 per cent, the average of twelve experiments being 95.6 per cent.

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The glycogen content of the brain of several species of animals as determined by the method described above is presented in Table I. The values obtained for rat brain by Asher and Takahashi (1924) approach the level we obtained for "resting" mammalian brain, but this appears to be the result of a balancing of errors, including loss of glycogen and contamination with non-fermentable reducing substances. These authors extracted "free carbohydrate" from the tissue with 60 per cent alcohol—a procedure which Holmes and Gerard (1929) state leads to loss of glycogen. Later recognizing that galactose liberated by the hydrolysis of cerebrosides had not been excluded, Asher and Takahashi adapted for quantitative purposes a method Rona and

TABLE I

Glycogen Content (Mg. per 100 Gm.) of Brain of Various Vertebrates

The brains were frozen *in situ* with liquid air under amytal anesthesia, with the exception of the sea turtle. The turtles were cooled in ice water, then decapitated. The head was split and the brain removed and crushed in KOH, the interval between decapitation and fixation being 2½ to 4½ minutes. The temperature of the tissue was 13°.

	No. of experiments	Maximum	Minimum	Average
Dog.....	7	130	77	102
Cat.....	5	101	77	86
Rabbit.....	5	99	70	82
Sea turtle.....	4	471	131	306

van Eweyk (1924) had used for the preparation of glycogen, and obtained values a third or half those secured with the Pflüger method (see also Takahashi (1925)). In our hands this modification yielded only 20 mg. per cent of glycogen as compared with 78 by our method.

SUMMARY

A modification of the Pflüger procedure is presented for determining glycogen in brain. The essential points of modification are: (a) avoidance of postmortem change in preparing the tissue for analysis; (b) rapid solution of the tissue by digestion with hot alcoholic potassium hydroxide; (c) separation of cerebrosides by

means of hot methyl alcohol-chloroform mixture; (d) correction for non-fermentable reducing substances liberated during acid hydrolysis.

By this method the recovery of glycogen added to brain averaged 95.6 per cent. Values from 70 to 130 mg. per 100 gm. are found in mammalian brain frozen *in situ*.

We are indebted to the Rockefeller Foundation for financial aid in carrying out this work.

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THE CARBOHYDRATE METABOLISM OF BRAIN

II. THE EFFECT OF VARYING THE CARBOHYDRATE AND INSULIN SUPPLY ON THE GLYCOGEN, FREE SUGAR, AND LACTIC ACID IN MAMMALIAN BRAIN

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A reexamination of the relationship of brain glycogen, free sugar, and lactic acid became necessary when it was demonstrated that autolytic changes take place in brain with such rapidity after death that "resting" levels for phosphocreatine and lactic acid can be secured only when the tissue is fixed *in situ* (Kerr, 1935; Avery, Kerr, and Ghantus, 1935). Moreover, the wide variation in values so far reported by various investigators for brain glycogen suggested defects in the methods used. In Paper I (Kerr, 1936) a modification of the Pflüger procedure was presented which permits the accurate determination of glycogen in nerve tissue. Using the technique of freezing the brain *in situ* with liquid air, and the improved method for glycogen, we have attempted to determine whether or not the carbohydrates of brain are subject to the same fluctuations as those of liver and muscle under a variety of influences, including overfeeding, fasting, phlorhizin poisoning, pancreatectomy, and overdosage with insulin.

EXPERIMENTAL

Methods

The brains were prepared for analysis as previously described (Kerr, 1935). Glycogen was determined in duplicate or triplicate by a method (Kerr, 1936) designed to avoid errors due to the presence of cerebroside. Lactic acid was determined in duplicate as described by Avery, Kerr, and Ghantus (1935) on zinc hydroxide filtrates, and the free sugar in the same filtrates by the

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TABLE I

Glycogen, Free Sugar, and Lactic Acid in Dog Cerebrum. Effects of Fasting, High Carbohydrate Diet, Infusion with Glucose, or Glucose with Insulin

Experiment No.	Conditions	Mg. per 100 gm. cerebrum				Blood sugar mg. per 100 cc.	Liver glycogen per cent
		Phosphocreatine P	Glycogen	Free sugar	Lactic acid		
10	Normal dog, uncontrolled diet	9.7	77	45	20.1		
11	" " " "	9.5	104	51	22.2		
12	" " " "	10.1	80	52	18.1		
16	" " " "	8.8	124	86	18.2		
17	" " " "	12.2	130	56	13.4		
18	" " " "	9.1	90	62	16.9		
20	" " " "	9.8	112	48	15.1		
138	" " " "		110				
139	" " " "		82				
147	" " " "		78				
150	" " " "		95				
Average.....		9.9	98	57	17.7		
81-B	Fasted 2 days		116	49	14.0		
37	" 2 "	8.6	100	55	21.6		
38	" 3 "	7.7	150	33	32.3		
36	" 3 "	8.4	96	71	21.4		
39	" 3½ "	10.4	135	48	26.4		
40	" 3½ "	8.5	142	47	23.8		
Average.....		8.7	123	51	23.3		
42	High carbohydrate-meat diet and glucose by mouth*		120	55	29.1		
43	" " "		113	87	19.4		
44	" " "		86	61	20.2		
35	5 min. after glucose infusion of 3.3 gm. per kilo	10.6	143	197	17.6	533	
67	30 min. after glucose infusion of 3 gm. per kilo	7.2	98	206	25.0		

* High carbohydrate-meat diet for 3 days and 1.5 gm. of glucose per kilo 2 to 3 hours before the brain was frozen.

TABLE I—*Concluded*

Experiment No.	Conditions	Mg. per 100 gm. cerebrum				Blood sugar	Liver glycogen
		Phosphocreatine P	Glycogen	Free sugar	Lactic acid		
68	90 min. after glucose infusion of 3 gm. per kilo	9.2	102	52	19.3		
73	5 min. after glucose-insulin infusion†	10.8	104	65	11.7	95	6.90
131	60 min. after glucose-insulin infusion†		118	206	16.9	223	2.42
80	135 min. after glucose-insulin infusion†		124	16	16.4	28	

* † 3 gm. of glucose and 3 units of insulin per kilo of body weight.

Shaffer-Somogyi (1933) procedure. Blood sugar was estimated by the Benedict (1931) procedure, with the exception of experiments numbered above 88, and in these the Folin (1929) procedure was used on unaltered blood filtrates. For phosphocreatine the Fiske-Subbarow (1925) method was used (see Kerr, 1935). This determination was included for the sake of ascertaining whether or not "resting" conditions were obtained during the fixation of the brain.

The experiments were conducted on dogs and rabbits, anesthetized intraperitoneally with amytal. The dosage was 60 to 65 mg. per kilo of body weight for dogs except the diabetic, phlorhizinized, and fasting animals, in which 50 to 55 mg. were found less apt to cause death. In rabbits 80 mg. per kilo were used.

Results

The cerebrum of dogs, selected at random with previous history unknown, was found to contain from 77 to 130 mg. per cent of glycogen, 45 to 86 mg. per cent of free sugar, and 13 to 22 mg. of lactic acid (see Table I). After fasting periods of 2 to nearly 4 days the glycogen content in three of the six animals was found to

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be even higher than in the non-fasting dogs, but whether this difference is significant is doubtful. A diet of rice, bread, and meat in excess for 3 days, followed by glucose (1.5 mg. per kilo) by stomach tube 2 to 3 hours before the animal was sacrificed produced no evidence of storage of glycogen in the brain, or other changes.

In a further effort to demonstrate whether or not glycogen could be stored in the brain, glucose infusions of 3 gm. per kilo of body weight were given to three dogs. A 25 per cent solution was introduced slowly into the femoral vein during a period of 40 minutes. The brains were frozen 5, 30, and 90 minutes respectively after the completion of the glucose infusion. In one of the three (Experiment 35, Table I) the glycogen level was found to be 143 mg. per cent (somewhat above the upper limit observed in the "normal" dogs), whereas in the other two (Experiments 67 and 68) the glycogen content was near the normal average. In Experiment 35 the glucose infusion raised the free sugar of the brain to 3 to 4 times the normal value, but less than half the blood level. Within 90 minutes after the infusion the brain sugar was again within normal limits (Experiment 68).

A combined glucose and insulin infusion (3 gm. of glucose and 3 units of insulin per kilo of body weight) administered intravenously over a 40 minute period likewise failed to increase the glycogen content of the brain above normal. In Experiment 73 the brain was frozen 5 minutes after the infusion, at which time the sugar of both blood and brain was at normal levels, the liver glycogen 6.9 per cent, and the brain glycogen (104 mg.) at the normal average. In Experiment 80 2½ hours elapsed between completion of the infusion and fixation of the brain, with the result that very low levels were reached for both blood sugar (28 mg.) and brain sugar (16 mg.). The glycogen content (124 mg.) was found to be within normal limits.

Depletion of the glycogen reserves of the body by removal of the pancreas failed to disturb the level of brain glycogen (Table II). In three of these experiments the glycogen of liver was lowered to 0.15 to 0.18 per cent, while that in brain remained well within normal limits. Under these conditions the free sugar of brain was greatly increased, although remaining lower than the blood sugar, and lactic acid was not significantly altered.

Combined fasting and phlorhizin poisoning of sufficient severity to reduce the glycogen of liver to the low level of 0.049 to 0.063 per cent failed to lower the level of brain glycogen below the normal (Table II). The free sugar was, however, lowered to nearly half the normal value, as was also the blood sugar.

TABLE II

Effect of Pancreatectomy and of Phlorhizin Poisoning on Glycogen, Free Sugar, and Lactic Acid of Dog Cerebrum

Experiment No.	Conditions	Interval between pancreatectomy and brain analysis	Urine sugar	Blood sugar	Liver glycogen	Mg. per 100 gm. cerebrum			
						Phosphocreatine P	Glycogen	Free sugar	Lactic acid
		days		mg. per 100 cc.	per cent				
61	Depancreatized (partial ?)		None	80	6.38	8.6	108	71	17.2
88	" "	9	"	117	6.79	7.3	117	77	25.2
95	" "	11	"				104		
87	Depancreatized	9	+	500	4.36	10.7	115	224	14.6
89	"	9	+	332	0.15	6.1	86	166	38.6
90	"	7	+	334	0.18	9.4	107	156	21.1
62-A	"		+	286	0.17	10.7	119	152	18.9
94	"	11	+	400	1.98		109	306	13.6
70	Phlorhizinized*			48	0.063	8.7	96	36	20.6
71	"			40	0.049	9.9	116	35	17.8
72	"			57	0.052	8.5	103	35	65.6

* These dogs were fasted for 7 days, injected subcutaneously with 1 gm. of phlorhizin emulsified in 10 cc. of olive oil on the 4th and 5th days, and with adrenalin (1 mg.) three times on the 6th day. On the 7th day the brains were frozen under amytal anesthesia.

The effect of insulin (15 to 20 units per kilo) on the brain glycogen of dogs is presented in Table III. In five of the eight experiments the glycogen was brought far below the lowest level observed in normal dogs (see Table I), in two others the lower normal limit was approached, and in one (Experiment 78) the

TABLE III
Effect of Insulin Overdosage on Glycogen, Free Sugar, and Lactic Acid of Dog Cerebrum

Ex- peri- ment No.	Fast- ing period	Insulin		Remarks	Amytal given	Brain frozen	Blood sugar	Liver gly- cogen	Mg. per 100 gm. cerebrum	
		Dose	Time						Gly- cogen	Free sugar
	hrs.	units per kg.					mg. per 100 cc.	per cent		
47	15	10	8.15 a.m.	Depressed	11.05 a.m.	11.20 a.m.	27		80	21
48	15	5	9.50 "	"	11.10 "	11.25 "	55		82	22
49	15	10	8.20 "		10.10 "	10.45 "	57		62	29
50	15	5	9.55 "	Convulsions 10.10 a.m.						
	15	10	7.50 "							
		10	7.55 "							
		5	12.05 p.m.	At noon unable to walk	2.50 p.m.	3.00 p.m.	34		41	24
78	19	5	2.05 "	No convulsions						
		10	10.02 a.m.	" symptoms						
		5	12.00 m.	Depressed, will not walk; no con- vulsions	3.15 "	3.38 "	21		99	15
		4	2.45 p.m.							
79	19	10	10.10 a.m.	Convulsions at 12.40 p.m.	12.45 "	1.15 "	39		47	20
111	23	5	12.00 m.	Unable to stand at noon; convul- sions at 1.06 p.m.	1.06 "	1.30 "	31	7.33	34	22
112	23	15	9.50 "	Convulsions at 1.40 p.m.	1.45 "	2.05 "	14	5.34	44	21

TABLE IV
Effect of Insulin Overdosage on Glycogen, Free Sugar, and Lactic Acid of Cerebrum of Rabbits

Experiment No.	Sex	Time fasted	Insulin		Remarks	Amytal given	Brain frozen	Blood sugar mg. per 100 cc.	Liver glycogen per cent	Mg. per 100 gm. cerebrum		
			Dose	Time						Glycogen	Free sugar	Lactic acid
		hrs.	units per kg.									
91	♂	?			Normal control			133	11.2	97	35	14
92	♂	?			"			116	8.2	70	52	17
93	♂	?			"			166	6.1	70	70	15
99	♂	0			"			105	6.1	76	53	9
100	♂	14			"			133	2.9	99	75	13
103	♀	16	10	9.05 a.m.								
			5	12.20 p.m.	No signs of depression	1.22 p.m.	1.36 p.m.	25	9.4	61	12	8
107	♀	17	10	10.15 a.m.	"	"	"	19	5.0	35	17	9
			5	12.23 p.m.	"	"	"					
109	♂	17	10	10.10 a.m.	"	"	"					
			5	12.28 p.m.	"	"	"					
101	♀	18	20	12.00 m.	Depressed, unable to sit up	1.58 "	2.10 "	21	1.8	62	13	11
					Muscular twitching at 11 a.m.; no convulsions	2.30 "	3.28 "	21	4.5	26	8	15
104	♂	18	20	9.00 a.m.	Convulsions at 11.50 a.m., again at 1.25 p.m.	11.02 a.m.	12.00 m.	7	1.4	34	13	9
97	♂	18	20	9.00 "	Convulsions at 11.50 a.m., again at 1.25 p.m.	1.25 p.m.	1.37 p.m.	25	6.2	29	12	17
98	♂	18	20	9.00 "	Convulsions at 11.32 a.m.	11.32 a.m.	11.45 a.m.	44	5.3	41	10	20
102	♀	18	20	12.25 p.m.	" " 2.30 p.m.	2.35 p.m.	2.58 p.m.	8	3.8	46	5	8
105	♂	18	20	9.15 a.m.	Unable to stand at 10.45 a.m. Violent convulsions at 10.58 and again at 11.15 a.m.	11.03 a.m.	11.23 a.m.	8	0.38	32	13	19
108	♂	17	10	10.00 a.m.	Convulsions at 1.20 p.m.	1.20 p.m.	1.50 p.m.	8	2.78	30	5	8
			5	12.25 p.m.								

concentration was about the average normal. In all ten experiments on rabbits (Table IV) insulin caused a sharp lowering of the glycogen level in brain.

Corresponding with the fall of blood sugar after insulin, the free sugar of brain was likewise diminished to concentrations far below the normal. In all but three cases (Experiment 112, Table III; Experiments 104 and 105, Table IV) the brain sugar was found to be considerably less than that of blood. No consistent changes in the lactic acid content of brain were caused by the various experimental conditions, with the exception that a high level was observed in one phlorhizinized dog (Experiment 77, Table II). A relatively low content of lactic acid was found in a number of rabbits, including the control group.

None of the experimental procedures caused significant changes in the phosphocreatine content of brain.

DISCUSSION

Glycogen—Brain resembles muscle in its ability to retain glycogen more tenaciously than liver. Neither fasting,¹ pancreatectomy, nor phlorhizin poisoning with adrenalin caused significant changes in the glycogen of brain, whereas all these conditions result in marked depletion in the liver. After pancreatectomy the glycogen of muscle, like that of brain, remains at a high level, even if food is discontinued (Chaikoff, 1927).

It appears also that the brain cannot store a significant surplus of glycogen, since the administration of glucose orally or intravenously with or without insulin failed to raise the glycogen level above that found in normal or fasting animals. Cori and Cori (1926, 1928, *a*) report a different behavior for muscle. They fed glucose to normal rats and observed a higher muscle glycogen in the animals injected with insulin than in the controls.

Whether the loss of brain glycogen caused by insulin overdosage is a direct effect of insulin or the result of a secondary output of adrenalin during the period of hypoglycemia remains to be deter-

¹ The fact that three fasting dogs had higher levels of brain glycogen than any of the controls, and that the average for the fasting animals was 25 mg. higher than the control group, suggests that the fuel reserve in the brain may be slightly increased, while that in the liver is greatly diminished. It is certain that the glycogen of brain is not depleted during 3 to 4 days fasting.

mined. That the lowering of muscle glycogen by insulin is actually an adrenalin effect was established by Cori and Cori (1928, b).

Our experiments confirm the results of Asher and Takahashi (1924) only in part. These authors concluded that the glycogen of brain remained unaffected by severe depletion of the carbohydrate reserves of muscle and liver. They concluded further that insulin caused an increase of glycogen if convulsions were avoided, but, if excitation of the central nervous system occurred to such an extent that convulsions resulted, glycogen was greatly decreased. In our experiments, however, the lowest glycogen found after insulin overdosage was noted in an animal which had no convulsions (Experiment 50). In view of the fact that these authors acknowledged that their failure to separate cerebroside resulted in values 2 to 3 times higher than they later found by a modified method, their evidence must be regarded as inconclusive. Winterstein and Hirschberg (1925), experimenting on the isolated central nervous system of the frog, concluded that insulin in high concentration caused a loss of glycogen, whereas small doses caused an increase. It has been pointed out elsewhere (Kerr, 1936) that the method they adopted for separating cerebroside from glycogen is inadequate, and possibly explains many of the very high values they obtained for "glycogen." The same criticism must also be made of the experiments of Kobori (1926) who used the methods of Asher and Takahashi (1924) and of Winterstein and Hirschberg (1925).

*Fermentable Sugar in Brain*²—On comparing the figures we obtained for brain with those of Cori, Closs, and Cori (1933) in rabbit muscle (non-fasting) under strictly "resting" conditions it appears that the quantity of free sugar in brain (35 to 75 mg. per cent for the rabbit) is nearly within the same range as that in

² The nature of the free carbohydrate of brain has not been conclusively demonstrated. Holmes (1929) prepared from extracts of crab nerve ganglia an osazone with a crystal form typical of glucosazone. He states, however, that in the extraction of free sugar from the tissue with 60 per cent alcohol some glycogen also dissolved and gave rise to high values for free sugar, hence the isolation of the osazone is of doubtful significance. Within the limits of error for determining the small quantities in question we find the reducing substance in the zinc hydroxide filtrate to be entirely fermented by yeast.

heart muscle (46 to 59 mg.) and much higher than in skeletal muscle (13 mg.).

The concentration of free sugar in brain, as in any other tissue, must be the resultant of a number of factors, including the rate of utilization as well as the concentration in blood and the volume flow of the blood. The influence of the blood glucose level is evident from the high concentration in the brain of diabetic dogs and the low level in the phlorhizinized animals (see Table II). A rapid rate of utilization is suggested by the fact that the concentration in brain is considerably less (half to two-thirds) than in blood, both in hypoglycemia and in hyperglycemia. After insulin the sugar concentration in brain is maintained at a considerably lower level than in blood in all but three of the sixteen experiments (Experiment 112, Table III; Experiments 104 and 105, Table IV). It should be noted that in these three experiments the blood sugar had reached the lowest points obtained. The explanation may be found in a similar situation noted by Cori, Closs, and Cori (1933) in rabbit muscle. These authors found a decrease in the fermentable sugar of skeletal and heart muscle during moderate insulin hypoglycemia, but in severe hypoglycemia there occurred a marked rise of muscle sugar, so that it equaled or even surpassed the plasma sugar concentration. This secondary rise was found to be due to adrenalin secretion.

The lowering of blood sugar after insulin is undoubtedly the result rather than the cause of a lowered tissue sugar concentration, since the tissue is the "vacuum" into which the sugar passes and disappears. The lowering of the free sugar and glycogen of brain appears to be due to an increased utilization of carbohydrate in the brain itself (or to conversion into other compounds) rather than to transport to other tissues, since the concentration of free sugar in brain is constantly less than in blood, and since extreme depletion of the carbohydrate reserves of the body by fasting and treatment with phlorhizin and adrenalin has no effect on brain glycogen.³ On the other hand, a secondary lowering of brain sugar could occur owing to insufficient supply from the blood, as in the hypoglycemia which follows hepatectomy. The disturbances of central nervous origin during insulin

³ The lowering of brain glycogen by insulin occurs while there is still a good reserve of glycogen in the liver, but this is probably not available, owing to inhibition of glycogenolysis by insulin (Cori, 1931).

hypoglycemia are possibly due to a carbohydrate deficit caused by both factors; *i.e.*, increased utilization by the brain and insufficient replacement from the blood. Although it is tempting to attribute the nervous disturbances and convulsions to the lowered content of free sugar or of glycogen, it should be noted that in several experiments low levels of glycogen and sugar were observed with no prostration or convulsions (see Tables III and IV). However, the symptoms of hypoglycemia are abolished by the amytal anesthesia; hence we cannot be certain of a lack of correlation between the chemical findings and the nervous disturbances.

SUMMARY

Glycogen, free sugar, and lactic acid were determined in brain frozen *in situ* with liquid air in amytalized dogs and rabbits under various experimental conditions.

The glycogen content of the cerebrum of normal animals was found to lie within the range 77 to 150 (average 98 mg. per 100 gm.) in well fed and fasting dogs, and 70 to 99 mg. (average 82) in rabbits. Fasting, overfeeding, glucose infusion with or without insulin, phlorhizin poisoning followed by adrenalin, and pancreatectomy all failed to cause significant changes in the brain glycogen.

Overdosage with insulin caused a marked decrease in the brain glycogen of dogs and rabbits.

The free sugar of brain in the control animals varied from 35 to 75 mg. per 100 gm. in rabbits and 45 to 86 mg. in dogs. Lowering of the blood sugar by phlorhizin poisoning or by insulin caused a corresponding decrease in brain sugar. Hyperglycemia caused by pancreatectomy or by administering glucose caused a rise of sugar in brain. The free sugar of brain was constantly lower than that of blood, except in extreme insulin hypoglycemia.

Neither lactic acid nor phosphocreatine of brain was affected significantly by any of the experimental conditions.

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A NOTE CONCERNING THE DETERMINATION OF IODINE

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In 1934 the method employed in this Clinic for the determination of iodine in blood was described (1). Since that time it has been in constant use in our laboratory routine and has been found to be quite satisfactory. Some of the clinical findings procured by the use of these procedures have been published (2). We have also used it for the determination of iodine in thyroid glands of guinea pigs in a new method of assay for the thyrotropic hormone of the anterior lobe of the pituitary gland (3). We have found that the method can be considerably simplified for use in determining the amount of iodine in the thyroid gland and that some of the modifications are applicable to the original procedure when used on blood. By making these changes, it is possible to dispense with the use of the Fresenius flask and to avoid the necessity of twice transferring the distillate quantitatively from one vessel to another.

The method as applied to the microdetermination of iodine in thyroid tissue is as follows: Fresh thyroid tissue weighing not over 100 mg. and not less than 5 mg. is placed in a 70 cc. nickel crucible with 0.5 to 1 cc. of a saturated solution of potassium hydroxide. This is heated over a Bunsen flame until the tissue disintegrates and is evenly distributed throughout the hydroxide. The crucible is then placed in an electric furnace at 100° and over a period of about 30 minutes the temperature is raised to 400°, when the crucible is immediately removed.

The fused mass is washed into a 50 cc. Claissen flask with not more than 25 cc. of water. The Claissen flask has had the auxiliary tubulation sealed, thus eliminating the use of a rubber stopper and also reducing the surface of condensation. Fig. 1 shows the

arrangement for distillation. The end of the tube leading from the condenser goes to the bottom of a 50 cc. extraction flask which contains water just sufficient to cover the end of the condenser tube, 0.2 cc. of a 3 per cent solution of sulfuric acid, and 0.2 cc. of a 10 per cent solution of sodium bisulfite. To the contents of the Claissen flask are added quickly 2 cc. of a 50 per cent solution of sulfuric acid, 1 drop of a 10 per cent solution of ferric sulfate, and

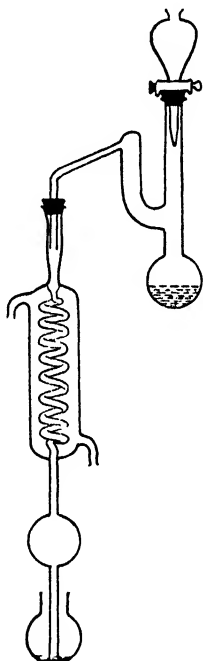


FIG. 1. Distillation apparatus used in microdetermination of iodine.

2 cc. of a 3 per cent solution of hydrogen peroxide (superoxol diluted to make a 3 per cent solution). More acid is added if necessary to make the solution definitely acid, as indicated by the presence or absence of ferric hydroxide. A glass bead is added to prevent bumping. The flask is immediately closed with the stopper and dropping funnel, the outlet of which has been drawn to a point. The contents of the flask are then boiled vigorously over a microburner, care being taken, however, to avoid flooding

of the side arm of the Claissen flask. One or two additional 2 cc. portions of 3 per cent hydrogen peroxide are added through the dropping funnel during the distillation. Heating is discontinued during these additions to avoid carrying hydrogen peroxide over with the distillate. The distillation is discontinued when the volume in the Claissen flask is reduced to about 5 cc. or when sulfates begin to crystallize on the side of the flask.

The extraction flask is placed on a wire gauze with asbestos center and the contents are boiled gently for 2 minutes to expel carbon dioxide and sulfur dioxide, a glass bead being used to prevent bumping. The solution is immediately made alkaline to litmus paper by the addition of a 10 per cent solution of potassium hydroxide; this should not require more than 3 drops. The solution is then carefully boiled down to a volume of 5 or 6 cc.; 1 drop of methyl orange is added, and the solution is neutralized by the addition of a 3 per cent solution of sulfuric acid. 2 drops of sulfuric acid in excess are then added along with 5 drops of bromine water, which should cause the solution upon shaking to turn yellow immediately. It is then boiled down very cautiously to about 2 cc., and cooled on ice. This should require between 3 to 5 minutes in order completely to remove excess bromine with minimum loss of iodine. 1 drop of a 1 per cent solution of starch and 2 drops of a 1 per cent solution of potassium iodide are added, and titration is carried out with a 0.001 N solution of sodium thio-sulfate which is delivered from a 0.2 cc. pipette graduated to 0.001 cc.

Methods employed for the purification of chemicals and for the calculation of the amount of iodine estimated are to be found in the former paper (1).

In determining the amount of iodine in blood, 10 cc. of blood are boiled with 12 cc. of a saturated solution of potassium hydroxide in a 300 cc. nickel crucible until foaming ceases. This requires less than 10 minutes. The crucible is placed in the muffle furnace at 250° for 30 minutes. The temperature is then increased to 360° over a period of 30 minutes. It is allowed to remain at 360° for 10 minutes, when the crucible is removed. Sufficient water is added to the fused mass to form a fluid paste which is extracted once with 25 cc. of 95 per cent ethyl alcohol and four more times with 10 cc. portions of 95 per cent ethyl alcohol. The

combined alcoholic extracts are placed in a 300 cc. nickel crucible with 0.5 cc. of a saturated solution of potassium hydroxide. The alcohol is evaporated on the steam bath, the contents of the crucible are dried gently over a free flame, and the crucible is placed in a muffle furnace at 385° for 15 minutes. The ash is then transferred to a 50 cc. Claissen flask and the procedure carried out as described above for the determination of iodine in the thyroid gland.

SUMMARY

Modifications and improvements in the method for the determination of iodine are described.

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A GRAVIMETRIC TECHNIQUE FOR THE DETERMINATION OF SMALL AMOUNTS OF PLASMA LIPIDS*

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In a study of the iodine numbers of plasma lipids Bloor's (1928) oxidation method for plasma lipids was used and proved very satisfactory. However, since the lipids had to be well purified in order to determine the iodine numbers, it was found simpler to weigh the purified lipids than to carry out the oxidative procedure. A gravimetric procedure was therefore developed, which has given very satisfactory results. The extraction and purification of the lipids are essentially the same as in Bloor's method. After weighing, the lipids may be dissolved in chloroform for the determination of cholesterol and iodine numbers.

For some reason gravimetry has been used very little for small amounts of plasma lipids, although the 20 to 30 mg. of lipid in 5 cc. of plasma are sufficient for accurate weighing on an ordinary analytical balance. Wilson and Hanner (1934), Kien and Wetzler-Ligeti (1935), and Wilson and Hansen (1936) have described gravimetric methods with 1 cc. of serum, but the use of a microbalance is required.

Method

Reagents—

Alcohol. 95 per cent alcohol is redistilled over sodium hydroxide.

Ether, redistilled over sodium bisulfite.

Petroleum ether. Commercial petroleum ether distilling be-

* The data in this article are taken from a thesis submitted to the graduate faculty of the University of Minnesota by Harold R. Street in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

tween 30–60° is washed with concentrated sulfuric acid by allowing the liquids to remain in contact for 2 or 3 days with occasional shaking, as described by Kirk, Page, and Van Slyke (1934). It is then redistilled.

Saturated potassium hydroxide solution.

Dilute sulfuric acid (1 volume of concentrated sulfuric acid to 3 volumes of distilled water).

Fat-free filter paper (extracted with ether).

Procedure

5 cc. of plasma are pipetted slowly and with vigorous stirring into 75 cc. of 3:1 alcohol-ether mixture (by volume) in a 100 cc. volumetric flask. (If less plasma is available, 2 or 3 cc. of plasma may be extracted with 40 cc. of the solvent.) The flask is immersed in boiling water until the solvent has boiled for a few seconds. The flask must be shaken vigorously to prevent explosive boiling. After standing at least 5 minutes, the contents of the flask are cooled to room temperature. The extract is filtered through fat-free filter paper into a 200 cc. Erlenmeyer flask and the protein residue in the funnel is washed with three quantities of alcohol-ether mixture.¹

To the extract and washings in the flask is added 0.75 cc. of saturated potassium hydroxide solution (or 0.4 cc. if only 2 or 3 cc. of plasma were taken for extraction). The solvent is evaporated *in vacuo* in a water bath at 78–82°.² It is necessary to watch the flask closely to prevent loss from foaming over as the last of the alcohol evaporates. Foaming is stopped by momentarily opening a stop-cock to relieve the vacuum. After disconnecting the flask the last traces of alcohol are swept out by a gentle current of air, continued till all odor of alcohol is gone. The evaporation requires 60 to 90 minutes with 100 cc. of extract.

3 cc. of dilute sulfuric acid are added to the flask; it is heated on the water bath for a minute and rotated so the acid reaches all parts of the flask where there is likely to be lipid. The lipids are then extracted from the acid suspension with four quantities of petroleum ether. The first extraction is made cold, but for the

¹ If it is desired to determine lipid phosphorus, the extract is made up to volume in a volumetric flask and aliquots are taken.

² The corks used for stoppering the flasks must be wrapped with tin-foil.

last three the flask is immersed in hot water and the contents are agitated for half a minute by gentle shaking. The petroleum ether is poured off as completely as possible from the aqueous layer and is received in a 50 cc. centrifuge tube. The volume of the extract should be 30 to 40 cc. The tube containing the petroleum ether extract is centrifuged a short time to throw down the non-lipid residue, and the extract is poured into a 50 cc. Erlenmeyer flask which has been partially immersed in water, wiped dry, placed in a desiccator for 2 or 3 hours, and weighed. The sides of the tube are washed with a few cc. of petroleum ether and after centrifugation this liquid is added to that in the flask. It is important that the last few drops of petroleum ether remain in the centrifuge tube each time, as a trace of dilute acid from the evaporation flask may be present.

The weighed flask containing the extract is placed in a water bath at 58–62° and the solvent is evaporated *in vacuo*.² When the evaporation is complete, the vacuum is relieved, the flask disconnected, and the last traces of petroleum ether are blown off with a gentle current of air continued for 30 seconds after all visible petroleum ether has been removed.

The flask is wiped dry, placed in a desiccator for 2 to 3 hours, and weighed.

If the values for cholesterol and fatty acids are desired, the lipids, after being weighed, are dissolved in chloroform, made up to volume in a volumetric flask, and aliquots are taken for the determination of cholesterol by Bloor's (1928) method. Fatty acids are calculated by subtracting the value for cholesterol from the value for total lipids.

DISCUSSION

Extraction of Lipids from Plasma—In the Bloor (1928) method of extracting lipids from plasma, which was used here, the plasma-alcohol-ether mixture is brought to boiling and kept at this temperature only a few seconds. Man and Gildea (1932–33) have claimed that the extraction performed in this manner is incomplete. They reported that in eight experiments from 5 to 31 per cent greater quantities of fatty acid were recovered when plasma was refluxed for 1 hour with alcohol-ether mixture than when aliquots of the same plasma were extracted by Bloor's method.

I have been unable to verify this finding. Several samples of dog plasma were extracted both by Bloor's method and by Man and Gildea's modification (5 cc. of plasma added to 75 cc. of solvent). In carrying out the extraction with Bloor's method the plasma and alcohol-ether mixture were allowed to stand 5 minutes after being brought to boiling, and were then cooled to room temperature. Total lipids were determined in each extract. With either method of extraction the results were always the same within experimental error. For instance, in one experiment two samples of plasma extracted by Bloor's method yielded 0.0161 and 0.0164 gm. of purified lipids, and two samples of the same plasma extracted by Man and Gildea's method yielded 0.0162 and 0.0163 gm. of purified lipids.

It is interesting to note that Stewart and Hendry (1935) found that the amount of fatty acids extracted was not increased by refluxing, but the number of carboxyl groups was increased because of oxidation. Since Man and Gildea determined fatty acids by acidimetric titration, this probably explains their higher values after refluxing. Boyd (1936) also found that refluxing plasma with alcohol-ether mixture did not increase the amount of lipid extracted.

Saponification has been used so that the weight of the fatty acids could be determined and because the mechanical difficulties are greater without saponification. A fine non-lipid residue appears, which is difficult to centrifuge out. Most of this residue dissolves in the dilute acid used after saponification.

Evaporation of Alcohol-Ether Extract—It is believed that the temperature of 78–82° used for the evaporation of the alcohol-ether extract is not too great in spite of certain statements appearing in the literature. In a discussion of the determination of plasma lipids, Kirk, Page, and Van Slyke (1934) state, "When the alcohol-ether solvent was evaporated at 60° or less, the re-extraction of the lipids yielded very constant results, much higher than those obtained if the beaker had been heated to 75°."

To determine whether a temperature of 78–82° is too high, a considerable quantity of alcohol-ether extract of dog plasma was obtained and several 50 and 100 cc. quantities of the extract were evaporated at 60–70° in a water bath and several at 90–92° on a steam bath. The evaporations were carried out in 800 cc. beakers.

The lipids were saponified with sodium ethylate during evaporation, acidified with dilute sulfuric acid afterwards, extracted with petroleum ether, and total lipids determined by Bloor's method. The same values were obtained with evaporation at either temperature. For example, two aliquots of alcohol-ether extract evaporated at 90–92° yielded plasma lipid values of 98.0 and 100.0 per cent of the correct amount, and two aliquots evaporated at 60–70° yielded plasma lipid values of 99.3 per cent of the correct amount. (The highest value in this series was taken as correct.)

It was concluded from these experiments that a temperature of 78–82° is not excessive for evaporation of alcohol-ether solvent under the conditions used. The discrepancy between these results and those of Kirk, Page, and Van Slyke (1934) may well be due to the fact that I have used saponification, whereas they have not. Extraction may perhaps be easier from the suspension of lipids in dilute acid after saponification than from the dry layer of lipids on the walls of the vessel after evaporation by the method of Kirk, Page, and Van Slyke.

Calculation of fatty acids has been made by subtracting the cholesterol value from the total lipids on the assumption made by Bloor (1928), that the plasma lipids after saponification consist entirely of fatty acids and cholesterol. This is not strictly correct, since, as shown by Lemeland (1921) and others, there is some non-saponifiable material in the plasma lipids in addition to cholesterol. However, this assumption serves well enough on the basis of the present knowledge of the composition of plasma lipids.

Accuracy of Method—In a study of plasma lipids, analyses of total lipids have been carried out in duplicate on twenty-five samples of plasma, 4 cc. of plasma being used for each analysis. The average deviation of each sample from the mean was 0.92 per cent, the maximum deviation was 2.67 per cent. This compares favorably with the accuracy of other micromethods for plasma lipids. Assuming the error to be doubled by halving the quantity of plasma, the error would still not be excessive if 2 cc. quantities of plasma were taken for analysis in place of 4 cc.

Comparison with Bloor's (1928) Method—Eight 2 cc. samples of dog plasma were analyzed gravimetrically for total lipids. The lipids were then dissolved in chloroform, cholesterol was deter-

mined colorimetrically by Bloor's (1928) method, and fatty acids were calculated by difference. Duplicate aliquots of the chloro-

TABLE I

Comparison of Gravimetric Method for Plasma Lipids with Bloor's Oxidative Method, with Dog or Human Plasma

Plasma No.	Cholesterol, colorimetric	Total plasma lipids			Total fatty acids*		
		Gravimetric	Oxidative	Per cent lower by oxidative method	Gravimetric	Oxidative	Per cent lower by oxidative method

Dog plasma							
	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.		mg. per 100 cc.	mg. per 100 cc.	
1	179	605	543	10.2	426	364	14.5
2	192	675	584	13.5	483	392	18.8
3	209	710	632	11.0	501	423	15.6
4	237	775	692	10.7	538	455	15.4
5	164	545	491	9.9	381	327	14.2
6	191	675	613	9.2	484	422	12.8
7	140	550	488	11.3	410	348	15.1
8	154	555	504	9.2	401	350	12.7
Maximum difference.....				13.5			18.8
Minimum ".....				9.2			12.7
Average ".....				10.6			14.9

Human plasma							
1	178	523	460	12.0	345	282	18.3
2	129	390	350	10.3	261	221	15.3
3	109	386	329	14.8	277	220	20.6
4	131	517	459	11.2	386	328	15.0
5	225	642	551	14.2	417	326	21.8
6	139	457	389	14.9	318	250	21.4
7	258	725	610	15.9	467	352	24.6
Maximum difference.....				15.9			24.6
Minimum ".....				10.3			15.0
Average ".....				13.3			19.6

* Fatty acids were calculated as total lipids minus cholesterol.

form solution were evaporated to dryness, and Bloor's oxidative procedure for total lipids was applied to this material. Total lipids were calculated with the use of the separate factors for

fatty acids and cholesterol instead of by the approximate formula. The results of the analyses by the two methods are given in Table I.

The values for total lipids determined with the oxidative method are consistently about 10 per cent lower than the values obtained with the gravimetric method. The differences between the fatty acid values are somewhat greater, since the difference is expressed entirely in the fatty acid fraction, cholesterol being the same.

A comparison of the two methods has also been made on human plasma. In this case a considerable quantity of alcohol-ether extract of plasma was made; duplicate aliquots equivalent to 4 cc. of plasma were taken for analysis by the gravimetric method and further aliquots for duplicate analyses by Bloor's (1928) method. The results are given in Table I. The differences in values for total lipids and fatty acids are in the same neighborhood as those found with dog plasma.

SUMMARY

A simple gravimetric method for the determination of total lipids in 2 to 5 cc. of plasma is described.

Bloor's oxidative method has been found to give values from 9.2 to 15.9 per cent lower than this gravimetric method.

I wish to express my thanks to Dr. George O. Burr and Dr. J. F. McClendon for their suggestions and criticisms in this work.

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PROTEIN AND WATER OF SERUM AND CELLS OF HUMAN BLOOD, WITH A NOTE ON THE MEASUREMENT OF RED BLOOD CELL VOLUME*

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In certain studies of the distribution of electrolytes in blood, both cells and serum were analyzed for water and protein. This paper deals with the relation of protein to water in the two phases of blood.

Since the proper allocation of solutes between cells and serum depends upon the exact separation and measurement of the red blood cells, special attention is given to the method employed in this study for the measurement of cell volume.

Methods

Whole blood and serum were separately analyzed. From the data thus obtained and measurement of the cell volume the concentrations of protein and water in the cells were calculated.

Blood was drawn without stasis under paraffin oil. Part of the blood was transferred to a sampling bulb over mercury and defibrinated anaerobically (1). This portion was used for whole blood analyses. Another portion was collected in centrifuge tubes containing oil, allowed to clot, and then centrifuged anaerobically (2). The supernatant serum was withdrawn and used for analyses.

Water was determined by means of dried weights. 2 cc. of whole blood or serum were placed in a small Pyrex dish and weighed. The dish was transferred to an electric oven and kept

* This study was made possible by a grant from the Research Fund of Yale University School of Medicine.

at a temperature of 93–97° until constant weight was attained. 48 hours usually sufficed for serum, 72 hours for whole blood. The water content was taken as the original weight of the blood or serum minus the final weight.

Oxygen capacity was determined on either defibrinated or oxalated whole blood by the carbon monoxide capacity method of Van Slyke and Hiller (3). Hemoglobin was calculated by multiplying the carbon monoxide capacity in volumes per cent by the ratio 167/224 (4).

Non-protein nitrogen was determined by the micro-Kjeldahl procedure.

Serum proteins were found by determining total nitrogen of serum by the macro-Kjeldahl procedure, superoxol being used to insure complete digestion. Non-protein nitrogen was subtracted from total nitrogen and the result multiplied by the protein factor 6.25. Where the non-protein nitrogen of serum was not directly determined, the non-protein nitrogen of whole blood multiplied by $\frac{2}{3}$ was used.

Whole blood protein nitrogen was similarly determined, with smaller aliquots ($\frac{1}{5}$ to $1\frac{1}{2}$ cc.). The dilutions were made in physiological saline, since water causes slight precipitation. Protein nitrogen of cells was multiplied by the factor 5.78 (5) to correct it to gm. of protein in terms of hemoglobin.

Measurement of Cell Volume

Various investigators have presented, for the concentrations of certain chemical constituents of the cell, values which have been obtained by the direct analysis of packed cells. Such analyses are difficult to make. They necessitate weighing the cells, which cannot be pipetted and measured volumetrically. The serum analyses, on the other hand, are made on specimens measured by volume. Since weight-volume relationships in cells are difficult to establish, direct comparisons of concentrations of the substances in the cell obtained from weighed quantities of cells and concentrations of the substances in the serum obtained from volumetrically measured amounts of serum are open to serious question. Furthermore, cell sludge, however washed or packed, must contain significant quantities of serum.

Given a reliable method for the estimation of cell volume, cell

values calculated from hematocrit, whole blood, and serum concentration values should be more accurate than those obtained from the direct analysis of cells.

Method—The hematocrit determinations were made by means of the ordinary Daland hematocrit tube fitted to a No. 1 International Equipment Company centrifuge. Centrifugation at high speed was continued until the cells were transparent, usually a matter of 45 to 60 minutes.

Some care is necessary in the selection and use of the tubes. As furnished by Bausch and Lomb, through the International Equipment Company, they have 100 graduations ± 10 . It is necessary to specify in ordering them that they contain 100 ± 1 graduations. Cutting down the graduations to 90 decreases the accuracy of the method. The tubes should then be calibrated by measuring them with a good cm. rule—checking total length, every tenth graduation, and start and finish of the graduations. By this means a correction factor for each tube is obtained. For instance, if the tube begins at 1 per cent instead of 0 and the total length in graduations is 99, the correction factor is: (observed reading - 1)/99. New tubes are carefully checked by actual cell volume determinations against old and tested tubes. The tubes must be scrupulously clean and neutral.

The centrifuge head for Daland hematocrit tubes supplied by the International Equipment Company is supposed to clamp the tubes by means of springs to prevent leakage. However, it has been found necessary to place elastic bands around the open ends of the tubes. Satisfactory bands can be cut from Gooch crucible tubing. The elastic bands should not be removed before reading.

The centrifuge head as supplied by the International Equipment Company accommodates only two tubes. Similar heads can easily be made to carry as many as eight tubes.

It should hardly be necessary to emphasize the fact that in order to obtain cell volume values, blood in a state of equilibrium must be used. It should be collected and preserved anaerobically, or else equilibrated with known tensions of CO_2 in air and then kept out of contact with air. After the addition of foreign substances, the blood should be allowed to stand in an anaerobic container for a sufficient length of time for equilibrium to be established.

If these directions are followed, the maximum error of the method is 1 volume per cent. This of course means that the percentage error will increase with decreasing cell volumes.

Relative Accuracy of the Hematocrit Method—Cell volume changes as determined by the hematocrit method were checked against serum protein determinations in three types of experiments.

1. Dry salt, sodium or potassium carbonate, or sodium or potassium chloride was added to one sample of blood, while an untreated specimen of the same blood was used as control. In these experiments the cell volume decreased.

2. The cells were caused to swell by the addition of water to whole blood.

3. The absolute cell volume was unaltered by the addition of isotonic sucrose solution, but the relative volume was decreased by dilution of the blood.

In each experiment a known amount of blood was centrifuged and the substance, salt, water, or sucrose solution, was added to the supernatant serum in order to obviate hemolysis. The cells and serum were then thoroughly mixed, and the mixture placed in a sampling bulb over mercury. A control sample of the same blood was treated in like manner: centrifuged, mixed, and placed over mercury. After allowing the specimens to remain in the sampling bulbs for 1 hour, shaking every 10 to 15 minutes, hematocrit tubes were filled from each bulb, and some of the blood from each bulb was centrifuged anaerobically (2) to obtain serum for analysis. In many cases a second measurement of cell volume was made on each specimen at least 1 hour later than the first, to prove that equilibrium had been attained at the time of the first cell volume estimation. In no case was there any change in cell volume in a given specimen after the longer time interval.

Salt and Water Experiments—The blood was treated as described above. In Table I the data concerning ratios of serum volume and ratios of serum proteins are given. Close agreements will be noted. This establishes the relative accuracy of the method.

Sucrose Experiments—40 cc. of blood were centrifuged and exactly 20 cc. of isotonic sucrose solution (9 per cent) were added to the supernatant serum. The subsequent procedure was identical with that described above. Whole blood was analyzed for nitrogen by the Kjeldahl method as a check on the dilutions.

TABLE I

Comparison of Ratios of Serum Volumes and of Serum Proteins

Substance added to blood	Serum volume ₁ Serum volume ₂	Serum protein ₁ Serum protein ₂	Substance added to blood	Serum volume ₁ Serum volume ₂	Serum protein ₁ Serum protein ₂
NaCl	0.940	0.938	H ₂ O	0.769	0.782
"	0.911	0.892	"	0.815	0.809
KCl	0.952	0.946	"	0.766	0.762
"	0.948	0.945	"	0.777	0.777
Na ₂ CO ₃	0.928	0.938	Sucrose	0.522	0.515
"	0.938	0.933	"	0.551	0.566
K ₂ CO ₃	0.967	0.963	"	0.482	0.474
"	0.920	0.922			

TABLE II

Sucrose Experiments

	Dilution	Observed serum volume	Calculated serum volume
		vol. per cent	vol. per cent
Control.....	1.50	56.3	70.9
Sucrose.....		71.9	
Control.....	1.50	63.0	75.3
Sucrose.....		76.2	
Control.....	1.51	48.2	65.7
Sucrose.....		66.2	

TABLE III

Comparison of Serum Solids and Proteins As Found by Dry Weight and As Calculated from Serum Volume

Substance added to blood		Solids of serum	Proteins of serum
		gm. per cent	gm. per cent
H ₂ O	Found	1.5	5.5
	Calculated	1.5	5.3
"	Found	1.4	6.0
	Calculated	1.2	6.0
Sucrose	Found	5.5	3.5
	Calculated	5.6	3.6
"	Found	5.0	3.8
	Calculated	4.9	3.7
"	Found	5.4	3.5
	Calculated	5.6	3.6

In Table II close agreement will be noted between the values calculated and found for the volumes of serum in the treated specimens. The absolute accuracy of the hematocrit method is thus demonstrated, since the addition of an isotonic solution to blood dilutes the number of cells by a calculated amount, without changing their absolute volume.

Other types of experiments carried on incidentally afford further corroborative evidence of the accuracy of the method.

In one type, dry weights of whole blood and serum were determined. Solids and protein of the original serum, if corrected for serum volume changes as determined by the hematocrit method and for added solids, agree with solids and proteins found by weight in the treated specimen (Table III).

In another set of experiments (Table I (6)), the addition of equimolecular amounts of the same sodium and potassium salts caused the same cell shrinkage as measured by the hematocrit method.¹

The third set of these experiments, presented in Table IV, was similar to the salt experiments described above. Total base was determined in whole blood and serum. Total base values for the cell were calculated with the hematocrit figures. Under the conditions of these experiments, no base crosses the cell membrane (6). Minimum transfers, less than the errors of the methods, are noted. The calculation of transfers from the concentrations of base in the serum is fairly direct and involves only the inverse ratio of serum volumes. The calculation of transfers from the concentrations of base in the cells is entirely dependent on the cell volume estimations, each of which is used twice. Nevertheless, there is agreement in the direction and magnitude of these transfers, calculated from the concentrations of total base in either serum or cells. If the hematocrit values should be in error by as much as 1 volume per cent, the agreement would be less exact. Obviously these experiments afford only the most indirect evidence of the accuracy of the hematocrit method, since the cell volume values were used in the establishment of the fact that no base

¹ If the cell volume method is accurate, there is no evidence in these experiments of such transfers of protein between cells and serum as Bellis and Scott (7) have claimed, in a recent article, follow the addition to blood of isotonic or hypertonic solutions.

TABLE IV
Comparison of Base Transfers As Calculated from Concentrations of Total Base in Cells and in Serum

Salt added to blood	m.-eq.	Salt recovered from whole blood	Cell volume per cent	Serum volume per cent	Ratio, serum volume: serum volume _s	Ratio, serum proteins: serum protein _s	Total base cells		Total base serum		Base transferred to	
							Found	Calculated	Found	Calculated	Cells	Serum
		m.-eq.					m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.
NaCl	37.3	39.2	47.6	52.4	0.872	0.882	100.8	120.3	150.6	196.5	0	0
"	36.6	37.1	47.5	52.5	0.896	0.904	101.3	120.5	147.3	192.2	+4.3	-3.0
KCl	37.1	35.6	47.4	52.6	0.854	0.854	117.8	135.6	141.8	188.8	-1.4	+1.2
"	36.6	35.2	47.1	52.9	0.892	0.883	112.3	130.0	145.4	189.0	-2.0	+1.4
Na ₂ SO ₄	38.0	37.3	46.8	53.2	0.913	0.928	112.8	128.0	146.2	197.4	+0.5	-0.3
			41.7	58.3			127.1	126.6	197.1			

crosses the red cell membrane. However, these and twelve other similar but less elaborate experiments do present evidence as to the extreme reliability of the method, since the consistent agreement manifest in the data can hardly be a coincidence.

The concentrations of water, protein, and hemoglobin in the cells were estimated by the general formula

$$\frac{(\text{Substance in whole blood}) - (\text{substance in serum } (1 - \text{cell volume}))}{\text{Cell volume}}$$

Results

The material analyzed consisted of 94 samples of blood from 73 persons, thirty normal adults and forty-three patients suffering from a variety of diseases.

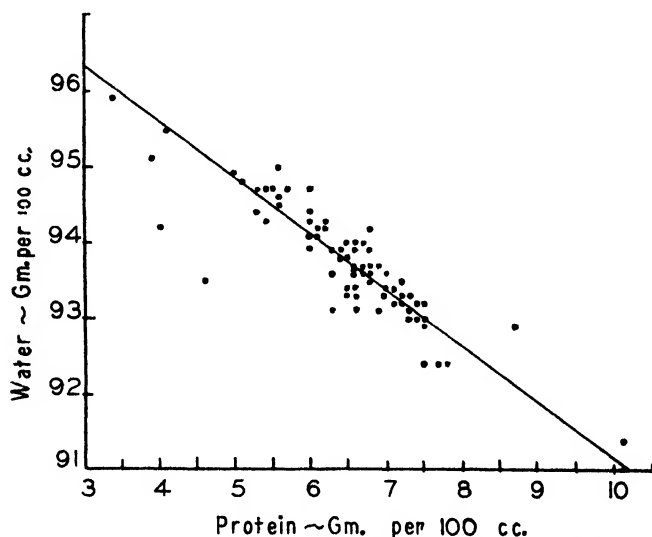


FIG. 1. The relation of protein to water in human blood serum. The line is defined by the equation $W_s = 98.5 - 0.745P_s$.

In Fig. 1 protein concentrations of the 94 sera are plotted against water content. There is an obvious correlation which seems to be linear. In theory the equation connecting these two functions could be expected to take the form,

$$W_s = 100 - (\alpha P_s + S)$$

in which W_s = cc. of water in 100 cc. of serum, P_s = gm. per cent of protein, S = volume per cent of non-protein solids, and α = a factor relating weight to volume, i.e. the specific molecular volume of the serum proteins. This equation can be changed to the form

$$W_s = A - \alpha P_s \quad (1)$$

in which $A = 100 - S$.

The equation of this form which best fits the experimental data is $W_s = 98.4 - 0.718P_s$, with a mean deviation of ± 0.24 and a standard deviation of ± 0.36 . The factor 0.718 is not, as theory demands, identical with that which is usually given as the specific molecular volume of the serum proteins, 0.745. The discrepancy may be referable to some systematic correlation between protein and other solids. Since, in clinical material of this nature, sera with low proteins are usually derived from patients with nephrosis, they generally contain an excess of lipids. It can be seen from Fig. 1 that two of the points with protein less than 5 per cent lie far below the line, and that none of the points in this range lies appreciably above the line. This indicates that these sera contain more than the usual volume of non-protein solids.

If the accepted factor for the specific molecular volume of serum proteins is used, the equation which best fits the data is

$$W_s = 98.5 - 0.745P_s \quad (2)$$

The difference between this and the empirical equation is negligible, less than ± 0.1 volume per cent of water between 0 and 10 per cent of protein. Either formula can be equally well employed for the estimation of serum water from protein concentration. Both will give falsely high values for W_s in sera containing unusually large amounts of lipids. However, no equation can be devised to escape this dilemma.

On the same principle employed for the estimation of the relation of protein to water in serum, the relation of protein to water in cells from 91² samples of blood was found to be defined best by the following equations.

² The results of three analyses were discarded because the values for cell water were, for inexplicable reasons, absurdly high.

$$W_c = 94.53 - 0.704Hb_c \quad (3)$$

(Mean deviation ± 0.903 ; standard deviation ± 1.24)

or, from proteins

$$W_c = 100.93 - 0.887P_c \quad (4)$$

(Mean deviation ± 0.85 ; standard deviation ± 1.68)

These curves (see Figs. 2 and 3) intersect at the point $Hb = 35$ volumes per cent, indicating that as Hb falls below 35 per cent, $P_c - Hb_c$, the non-hemoglobin protein, steadily increases. It might be expected, of course, that $P_c - Hb_c$, representing presumably the stroma of cells, would be related to the volume of the cells rather than to hemoglobin. It would, therefore, in equations of the form of Equations 3 and 4, appear to vary inversely as Hb_c . It could not, however, become a negative quantity above 35 per cent hemoglobin, as these equations suggest. Furthermore, Equation 4, as a theoretical expression, must be regarded with suspicion because the constant, 100.93, which should represent 100—non-protein solids, is greater than 100.

Again one might employ the specific molecular volume of hemoglobin, estimated at 0.75 (8, 9), in which case the best equation would be $W_c = 96.0 - 0.75Hb_c$. The mean deviation from this equation is ± 0.93 , standard deviation ± 1.30 , only slightly larger than the deviations from the empirical equation. This curve still intersects that derived from protein, albeit at a higher concentration of hemoglobin.

These peculiarities in the equations probably cannot be attributed entirely to analytical errors because the deviations from the curves are altogether too consistent and are not conspicuously greater in bloods with low hemoglobin, in which errors of analysis and calculation would be exaggerated.³ It seems probable that the difference between the slopes of the two equations is an expression of fact. The actual positions of both are dependent upon the values chosen for the molecular weight of hemoglobin and the nitrogen content of hemoglobin and of the proteins of cellular stroma, values which are comparatively rough approximations.

³ The concentration of hemoglobin in whole blood is not in this series related to the hemoglobin concentration in cells, since the bloods were taken from normal subjects, patients with polycythemia, and patients with anemias of all varieties.

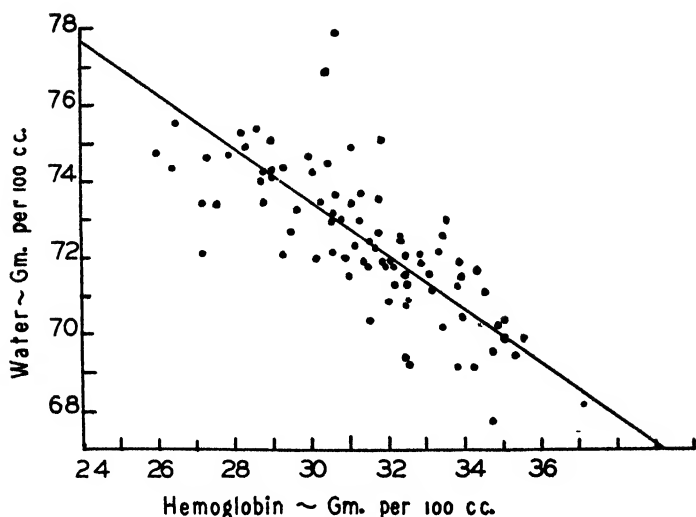


FIG. 2. The relation of hemoglobin to water in human red blood cells. The line is defined by the equation $W_e = 94.53 - 0.704Hb_e$.

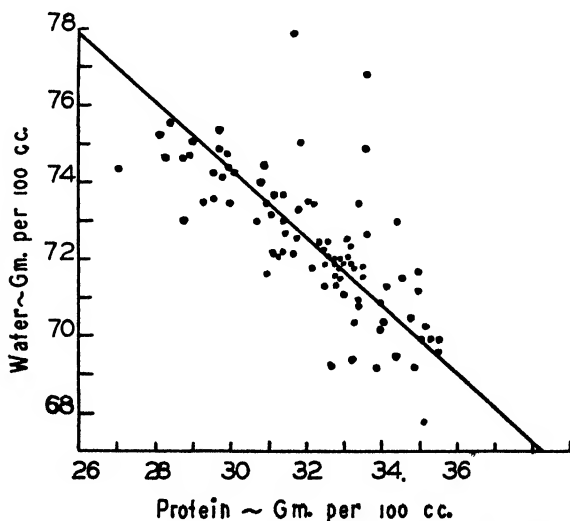


FIG. 3. The relation of protein to water in human red blood cells. The line is defined by the equation $W_e = 100.93 - 0.887P_e$.

The water of cells varies greatly, in these bloods from 67 to 80 per cent. The largest amounts were found, as might be expected, in bloods from patients with secondary anemia. Although the relative quantities of stroma protein in these cells seem to have been greater, they obviously do not compensate for the deficiency of hemoglobin. Nor do other solids appear to play a compensatory rôle. The widely accepted thesis, that the proportions of water to solids or proteins in cells remains constant, obviously does not hold for red blood cells. If secondary anemia may, by a stretch of the imagination, be looked upon as a specific form of malnutrition, the high water content of cells in this condition affords an example of the maintenance of form at the expense of constitution. The danger of drawing too close an analogy between the red blood cell and tissue cells is, of course, recognized. The red blood cell is both highly specialized and transitory. The dilution which characterizes secondary anemia must be considered as a congenital characteristic, while malnutrition in other cells is acquired. If only the thirty-three bloods from the thirty normal subjects are considered, the mean concentration of hemoglobin is 32.7 gm. per 100 cc. of cells (average deviation ± 1.1 , standard deviation ± 1.9 gm.); the mean concentration of protein is 33.1 gm. per 100 cc. of cells (average deviation ± 1.0 , standard deviation ± 1.9 gm.).

No attempt has been made to review the literature dealing with estimations of the amount of non-hemoglobin protein in blood, because all these estimations depend upon the choice of factors for the nitrogen content of hemoglobin.

SUMMARY

On the basis of 94 examinations of blood from thirty normal adults and forty-three patients equations have been derived which define the relations between water and protein in serum and hemoglobin in cells, and water and protein in cells.

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THE ACTION OF HYDROGEN PEROXIDE ON L-XYLOKETOSE (URINE PENTOSE)

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Previous experiments have indicated that *l*-xyloketose is more active chemically than other sugars commonly found in urine. Lasker and Enklewitz (1) have shown that pentose urine reduces Benedict's sugar reagent within 10 minutes at 55° and, based on this observation, have devised a simple test for the detection and estimation of *l*-xyloketose in urine.¹ Schmidt and Treiber (2) have prepared *d*-xyloketose and report that reduction of Fehling's solution occurs within 10 minutes at room temperature. Further proof of the chemical activity of *l*-xyloketose is to be found in the rapidity and ease with which it is oxidized by hydrogen peroxide in the cold.

The addition of hydrogen peroxide to pentose urine results in the rapid loss of its reducing properties. 1 cc. of 3 per cent hydrogen peroxide was added to 5 cc. of pentose urine; the solution was vigorously shaken for 2 minutes and then tested with Benedict's qualitative solution. Reduction of the alkaline copper solution no longer occurred. At higher temperatures the reaction was instantaneous. After treatment with hydrogen peroxide urine pentose no longer formed an osazone. Hydrogen peroxide reacts with phenylhydrazine; it was therefore necessary to remove the former from solution by titration with potassium permanganate, after which osazone formation was attempted in the usual manner. As a control, untreated pentose urine, similarly titrated with permanganate, was found to retain its reducing properties and on treatment with phenylhydrazine formed the characteristic osazone.

After treatment with hydrogen peroxide the urine still gave a positive test with Bial's reagent even though it no longer reduced

¹ We have identified *l*-xyloketose in forty-three different cases of pentosuria.

Benedict's solution. This is due to the greater sensitivity of Bial's reagent which reacts positively with pentose solutions of 0.01 per cent concentration, whereas Benedict's solution is no longer reduced when the concentration of sugar is less than 0.1 per cent.²

The following sugars were prepared in aqueous solution and in urine in concentrations similar to that found in pentosuria (0.5 per cent or less)—glucose, fructose, lactose, maltose, galactose, xylose, and arabinose. These were treated with peroxide at room temperature and it was found that they still retained their reducing properties when tested 72 hours later. These sugars do not react with hydrogen peroxide in the cold.

Pure *L*-xyloketose was prepared from the urine of pentosuric patients according to Greenwald's (3) method, and it was found that aqueous solutions of 0.5 per cent concentration were much less active than pentose urine. At room temperature treatment with 30 per cent peroxide for a period of an hour or longer was required before there was no further reduction; *D*-xyloketose, prepared according to the method of Schmidt and Treiber (2), was found to behave in a similar fashion. The addition of small amounts of ferric chloride speeded up the reaction, as did the addition of either normal or pentose urine. Apparently both normal and pentose urines contain catalysts which shorten the interval before reduction ceases. The addition of sodium cyanide did not inhibit or retard the oxidation.

Potassium persulfate and potassium dichromate were even more efficient than hydrogen peroxide in the oxidation of urine pentose. The addition of a pinch of either to such urine at room temperature resulted in the immediate loss of reducing properties, whereas the other sugars previously mentioned were unaffected and continued to reduce.

It was thought that the oxidation of urine pentose could also be accomplished by using ozone, but it was found that prolonged treatment with ozone was without effect and reduction of Benedict's solution still occurred.

² On several occasions we have examined samples of dilute urine from known cases of pentosuria and have obtained a positive Bial's test and a negative test with Benedict's qualitative solution. This is particularly true of infants and children who excrete much smaller amounts of pentose.

The remarkable chemical activity of urine pentose can best be illustrated by comparing it with the next active sugar, fructose, which reduces Benedict's solution within 30 minutes at 55°. Fructose urine does not react with peroxide in the cold; tested 1 week later, reducing bodies could still be demonstrated. An aqueous solution of 0.5 per cent, treated with large amounts of 30 per cent peroxide at 100°, requires about 24 hours before reduction ceases. At this time the solution is no longer optically active, gives a negative Seliwanoff test, and manifests an increased acidity, requiring much more alkali for neutralization.

In passing, it may be mentioned that ascorbic acid reduces Benedict's solution very quickly at ordinary temperatures and, like xyloketose, it loses its reducing properties on treatment with peroxide.

The oxidation of urine pentose by hydrogen peroxide at room temperatures can be utilized as a confirmatory test for this sugar in the following way: To 5 cc. of the urine from a suspected case 1 cc. of ordinary 3 per cent peroxide is added, the solution is well shaken for 2 minutes, and then tested for reduction with Benedict's qualitative solution. If there is no reduction, the urine sugar is xyloketose. Conversely, if reduction still occurs, xyloketose is excluded.

The identity of the oxidation products is being investigated.

SUMMARY

The addition of 3 per cent hydrogen peroxide to pentose urine at room temperature results in the rapid loss of reducing properties. Potassium persulfate and potassium dichromate are equally effective as oxidizing agents. Pure xyloketose in aqueous solution is not as active, requiring a longer interval and the more concentrated 30 per cent peroxide. Solutions of other sugars in urine and in water are unaffected by treatment with peroxide in the cold. The oxidation by peroxide can be used as a confirmatory test for the presence of xyloketose in urine.

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X-RAY DIFFRACTION PATTERNS OF CRYSTALLINE TOBACCO MOSAIC PROTEINS

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PLATE 1

(Received for publication, July 20, 1936)

The isolation (1) from mosaic-diseased plants of a crystalline protein that apparently carries all the biological characteristics of the infecting virus has aroused especial interest in its physical and chemical properties. We have made a series of x-ray diffraction studies to determine (1) whether this protein gives the same kind of sharp line x-ray pattern that is provided by inorganic crystals and (2) to ascertain whether there are differences in x-ray pattern corresponding to the different virus strains known to exist.

The x-ray equipment (2) used for this purpose was the same as that employed in preparing powder photographs (3) of other crystalline proteins. It consisted of a gas type x-ray tube with a chromium target capable of day and night operation at a power consumption somewhat in excess of a kilowatt. Such a powerful x-ray source is required because very fine pinhole slits are needed for recording the very long spacings present in protein crystals. With 0.15 mm. diameter pinholes and 15 cm. radius cameras the exposure times in the present experiments ranged between 24 and 48 hours. The diffraction cameras were of a vacuum type that could be filled with helium carrying the necessary water vapor content. It is essential to fill long radius cameras with helium (or hydrogen) because air scatters enough of the soft chromium K radiation to blacken the recording film. A suitable amount of water vapor must be present if crystalline diffractions are to be observed, since the tobacco mosaic virus protein crystals, like nearly all the crystalline proteins with which we have worked, contain much water of crystallization. If put into too dry an

atmosphere, such crystals will effloresce and the regularity of their internal structure will be destroyed. In too wet an atmosphere they will absorb water and go into solution. A suitable moisture content for the tobacco mosaic proteins was obtained by allowing helium to stand over the solution from which the crystals separated out. This is a simple humidifying scheme that is generally useful in the x-ray study of proteins. The sample to be photographed was prepared by placing a few drops of a heavy suspension of the crystals in a cold room, on a sintered glass filter to which suction had been applied. The pasty mass thus obtained was quickly inserted into a 2 mm. hole in the thin metal or celluloid plate that served as specimen holder. x-Ray transmission was through the crystal layer thus produced; its thickness, which must depend on the kind of radiation and the habit of the crystals being studied, could be varied by using different specimen holders.

We are indebted to Dr. W. M. Stanley of the Division of Animal and Plant Pathology of this Institute for the crystalline proteins studied. Accurate and detailed examination has been made of the proteins from plants infected with two strains of the tobacco mosaic disease—the ordinary strain and the more virulent aucuba strain. Solutions of the crystalline preparations which were photographed were biologically active—the ordinary protein producing the regular tobacco mosaic and the aucuba protein producing the yellow mottling characteristic of the aucuba mosaic disease.

The type of x-ray powder photograph obtained from either of these two crystalline proteins prepared by ammonium sulfate precipitation is shown in Fig. 1. This pattern, though inferior in clearness to those obtained from some proteins, contains the many sharp diffraction lines that characterize a true crystal. It seemed probable that the lack of clarity in these pictures might be due to strong background scattering by relatively large amounts of adherent ammonium sulfate solution. This possibility was tested in two ways. A suspension of the crystals, as precipitated with $(\text{NH}_4)_2\text{SO}_4$, was adjusted to a pH near the isoelectric point and dialyzed against acidified water till the $(\text{NH}_4)_2\text{SO}_4$ content was reduced to a negligible value. In another experiment carefully purified protein was recrystallized from solution by cautious acidification to about pH 4.5. Both the dialyzed and the acid-

precipitated crystals gave excellent powder photographs (Fig. 2) showing the same lines as the $(\text{NH}_4)_2\text{SO}_4$ -precipitated material but free of the partially obscuring background (Fig. 1). From this result it is necessary to conclude that these crystalline proteins bearing virus activity yield exactly the kind of x-ray diffraction pattern to be expected from any well crystallized protein.

TABLE I

Principal x-Ray Powder Lines of Crystalline Tobacco Mosaic Proteins

Ordinary strain		Aucuba strain		Ultraviolet irradiated protein Spacing
Spacing	Intensity	Spacing	Intensity	
Å.		Å.		Å.
80	Strong	80	Strong	
53	Medium	55	Medium	
37	Faint	37	Faint	
28	Very faint	27	Very faint	
20.8	Medium	21.0	Medium	21.2
16.2	Very faint	14.2	Very faint	14.7
14.2	" "		" "	
11.0	Strong	11.0	Strong	11.3
10.2	Faint	10.2	Faint	
9.2	Medium	9.2	Medium	9.2
7.44	"	7.43	"	7.5
6.5	Faint	6.5	Faint	
5.7	Very faint			
5.44	Faint	5.41	"	
4.95	Medium	4.95	Medium	
4.71	"	4.68	"	
4.44	"	4.45	"	4.46
4.08	Faint	4.09	Faint	4.10
3.88	Medium	3.88	Medium	3.88
3.70	Very faint			
3.54	" "			
3.39	Faint	3.39	Faint	

A series of experiments was also made to see if any alteration or improvement in diffraction pattern would result from repeated recrystallizations. Nine recrystallizations, according to the method of Stanley (4), were made and x-ray pictures taken at the outset and after the first, third, fifth, and ninth precipitations. These patterns could not be distinguished from one another.

Especial interest attaches to the question of whether or not the proteins corresponding to the two strains give identical x-ray patterns. As the data of Table I make evident, there are no observable differences either in the positions or in the intensities of lines produced by the ordinary and the aucuba proteins. These proteins are not identical, as shown not only by their differing biological activities but by some of their physical properties; the remarkable similarity in their x-ray patterns in the entire range between 80 Å. and 3 Å. is comprehensible only if their molecules in the solid state have shapes that differ in only minor details.

It is worthy of note that no spacings are to be found in the region between 80 Å. and 125 Å. This larger spacing is the longest that can be recorded in the experimental arrangement used. Inasmuch as ultracentrifugal analysis¹ points to a molecule that well might have spacings in excess of 125 Å., the recorded 80 Å. diffraction should not be taken as a final upper limit until equipment has been developed which permits an exploration for still greater spacings.

An x-ray photograph has also been made of a crystalline protein obtained by irradiating with ultraviolet light a solution of tobacco mosaic protein until it had lost its virus activity. Too few crystals were available for the best photography, but, as Table I indicates, the strongest diffraction lines of this altered protein are the same as those of the original crystalline material.

SUMMARY

A series of x-ray powder diffraction photographs has been made of crystalline tobacco mosaic virus proteins. The patterns thus obtained, with many sharp reflections between 80 Å. and 3 Å., are exactly those to be expected from true crystals composed of large molecules. No differences could be found in the patterns of the proteins of the ordinary and of the aucuba strains of the disease. Neither was there any alteration in the x-ray photograph after nine successive recrystallizations. Tobacco mosaic virus protein completely inactivated by means of ultraviolet irradiation and subsequently crystallized gave a photograph having principal diffraction lines that are the same as those of the active protein.

¹ Results of such a study will shortly be published.

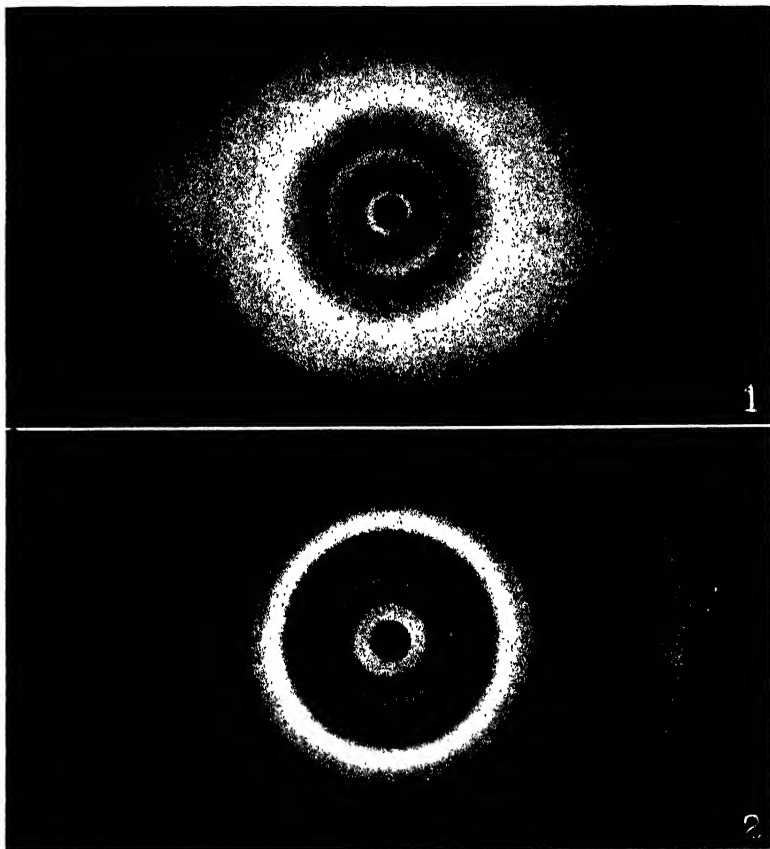
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EXPLANATION OF PLATE I

FIG. 1. x-Ray diffraction pattern of paste of $(\text{NH}_4)_2\text{SO}_4$ -precipitated tobacco mosaic virus protein crystals. Chromium K radiation. Camera radius = 7.4 cm.

FIG. 2. x-Ray diffraction pattern of acid-precipitated tobacco mosaic virus protein crystals. Chromium K radiation. Camera radius = 7.4 cm.



(Wyckoff and Corey: x-Ray patterns of mosaic proteins.)

THE PREPARATION OF EXTRACTS CONTAINING THE ADRENAL CORTICAL HORMONE

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Since the report by Rogoff and Stewart (1) that the life span of adrenalectomized dogs could be prolonged by the administration of adrenal cortical extracts, a number of methods for preparing active extracts have been published (2-7). Many of these procedures are well adapted for preparing potent extracts on a small laboratory scale. However, in attempting to apply them on a larger basis for the preparation of sufficient quantities of adrenal cortical hormone for clinical use and for fractionation studies, we very early found that many of the steps were difficult to apply and were often accompanied by considerable losses of hormone.

One of the chief difficulties in preparing adrenal cortical extracts from whole adrenal glands has been the removal of toxic impurities, particularly epinephrine and its decomposition products. The permutit step as employed by Swingle and Piffner (3) is capable of removing large quantities of epinephrine, especially if repeated several times. However, this operation is somewhat difficult to adapt to the filtration of large volumes of extract, and the alternative procedures, as suggested by Kendall and coworkers (8) and Grollman and Firor (6), involving extraction with dilute acids or alkalies, have, in our experience, resulted in a considerable loss of cortical hormone.

The present study was undertaken to develop a more simplified procedure for making active extracts sufficiently purified for clinical use and readily adaptable to large scale manipulation.

EXPERIMENTAL

Assay of Extracts—Adrenalectomized rats and dogs have been used; a comparative study of these two methods of assay constitutes a separate report from this laboratory. The rat unit is

defined as the minimum daily dose of extract which, administered by single subcutaneous injection to 4 week-old rats weighing 50 to 60 gm., over a period of 20 days, is sufficient to maintain life in 80 per cent of the animals and permit an average growth of 20 gm. per rat for the 20 day period. The average survival of uninjected controls is 6 to 7 days, and the animals injected with sustaining amounts of hormone for 20 days have been observed to die in an average of 7 to 8 days after cessation of injections. Out of 675 adrenalectomized rats used in these studies only nineteen have shown indefinite survivals without extract and in most of these the presence of residual cortical tissue could be demonstrated upon autopsy. The rat method is somewhat less time-consuming than the dog method and has routinely been used for following the potency of the various intermediate fractions. A fraction was called inactive if, injected in daily doses representing 12 gm. of gland, it failed to lengthen the survival period in a group of at least five rats as compared to that of five uninjected controls.

The final extracts have also been assayed by the dog method according to the procedure of Harrop, Pffner, Weinstein, and Swingle (9). Either of these methods is capable of giving satisfactory results, although the hormone requirement of immature growing rats is relatively much greater on a per kilo basis than that of adult adrenalectomized dogs.

Starting Material—Whole beef adrenal glands were used in all of these studies. The glands were frozen at the packing house and shipped to the laboratory packed in dry ice. In every case the glands were finely chopped while frozen and transferred immediately to the initial solvent.

Preparation of Extracts—Preliminary experiments were concerned with studying the various published methods for preparing adrenal cortical extracts; 3 to 4 kilo quantities of gland were used and the end-fractions assayed by the rat method. These experiments indicated that we were able to obtain the best yields of hormone by the procedure of Swingle and Pffner (3). Consequently, this method was used as a basis of comparison in our attempts to develop a more simple and effective procedure for obtaining a product of maximum purity in a minimum number of steps.

For initial extraction of the glands a water-miscible solvent

offers definite advantages over a non-miscible solvent such as ether, employed by Hartman and coworkers (2). Swingle and Pfiffner (3) have used alcohol as an initial solvent. A comparison of acid acetone as recommended by Kendall and McKenzie (7) with neutral acetone as employed by Kutz (5), Grollman and Firor (6), and others showed that acid acetone extraction gave poorer yields of hormone and a less pure product.

Neutral acetone was next compared with neutral alcohol in parallel extractions of 3 kilo aliquots of the same lot of glands. Alcohol yielded 44.7 gm. of extracted solids per kilo of fresh gland, whereas acetone yielded 31.6 gm. of extracted solids per kilo of gland. When these two lots were finished by the method of Swingle and Pfiffner (3), the yields of hormone by the rat method were found to be equivalent. The acetone extract contained much less phospholipid than was obtained in an alcohol extract. Consequently acetone is a more effective solvent than alcohol in that it yields a first extract of greater purity without sacrificing hormone yields.

The acetone extract was next concentrated *in vacuo* below 45° to remove acetone. The aqueous residue so obtained contains considerable quantities of inert fatty material. Solvents such as benzene or ether will remove this fatty material but also will extract the cortical hormone almost quantitatively. Consequently, the use of such a solvent at this step yields an active hormone extract from which the inert lipid contaminants can be removed only with considerable difficulty. However, it was found that extraction of the aqueous residue with petroleum ether would remove large quantities of fat, leaving all of the cortical hormone in the aqueous phase. Thus, the use of petroleum ether here accomplishes the separation of a large amount of impurities, making subsequent extraction of the hormone from the aqueous phase much easier.

The next step was to choose a solvent which would extract the cortical hormone quantitatively from the aqueous solution and remove only a minimum quantity of extraneous substances. Benzene is a good solvent for the hormone but it also removes considerable quantities of phospholipids and epinephrine. If benzene is used at this point, two additional steps must be introduced for the removal, respectively, of phospholipids and epi-

nephrine. However, it was found that ethylene dichloride is a highly selective solvent, since it extracts from the aqueous solution all of the cortical hormone but only a minimum of inert solids and no significant amount of phospholipids or epinephrine. The specificity of ethylene dichloride with respect to the exclusion of these undesirable substances is shown by the fact that the hormone-free aqueous fraction remaining after ethylene dichloride extraction will yield to subsequent benzene extraction considerable quantities of phospholipids and epinephrine. The biologically active ethylene dichloride fraction, on the other hand, contains no phospholipid which can be precipitated by cold acetone and the epinephrine content is so low that no further steps are required for its removal. Thus, the simple expedient of using ethylene dichloride for extraction of the cortical hormone from the petroleum ether-washed aqueous residue has made possible the elimination of the specific steps for the removal of phospholipids and epinephrine which have been so troublesome in previously described procedures.

The ethylene dichloride, although it separates quickly from the aqueous solution, carries with it a very small amount of water and water-soluble substances which can be removed by chilling to -15° and filtering from the separated ice. The small amount of cholesterol and neutral fat still present in the ethylene dichloride solution can be removed by partitioning between aqueous alcohol and petroleum ether. This can best be done by using the fractional method as described by Butenandt (10) who used this step in the purification of theelin fractions from pregnancy urine. The ethylene dichloride solution from 100 kilos of glands is concentrated *in vacuo* to remove the solvent and the residue is dissolved in 200 cc. of ethyl alcohol. An equal volume of petroleum ether, b.p. $30-70^{\circ}$, is added and mixed. Sufficient water is then added to make the alcohol 90 per cent, which causes the separation of a part of the petroleum ether, which is removed. The addition of water sufficient to reduce the alcohol concentration from 90 to 80 per cent causes the separation of a second portion of the petroleum ether. Finally, the alcohol phase is reduced to 70 per cent alcohol by the addition of water and extracted thoroughly with petroleum ether. When the distribution was done in this way, little trouble was encountered with emulsions.

The 70 per cent alcohol solution containing the cortical hormone is concentrated *in vacuo* below 45°, leaving an aqueous colloidal solution. This solution upon cooling and after addition of NaCl to make 0.9 per cent deposits an insoluble tarry substance which is inactive. It was found that a concentration of 10 mg. of extractive solids per cc. was optimum for the precipitation of this substance. The pH varied from 4.5 to 5.0. After the removal of this inactive precipitate by centrifuging, the clear supernatant solution is decanted and diluted with 0.9 per cent

Flow Sheet of Fractionation Procedure for Preparation of Adrenal Cortical Hormone

100 kilos of whole beef adrenals		
Extract with acetone: 250 liters, 99%; 200 liters, 80%		
Acetone extract, 3500 gm. Concentrate to 80 liters and extract with petroleum ether: 20 liters, 20 liters		Gland residue (discard)
Aqueous fraction, 2000 gm. Extract with ethylene dichloride: 40 liters, 40 liters, 40 liters		Petroleum ether-soluble, 1500 gm. (inactive, discard)
Ethylene dichloride-soluble, 4.8 gm. Solvent removed and fraction partitioned between 70% alcohol and petroleum ether		Aqueous solution (inactive, discard or use for recovery of epinephrine)
70% alcohol-soluble, 3.7 gm. Alcohol removed and NaCl added to 0.9%		Petroleum ether-soluble, 1.1 gm. (discard)
Aqueous solution. Make volume up to 2500 cc. adding NaCl to make 0.9% and alcohol to make 10%. Sterilize by Berkefeld filtration. Total gland extractives, 1.8 gm. Assay, 250,000 dog units		Precipitate, 1.9 gm. (inactive, discard)

saline, so that each cc. represents 40 gm. of fresh whole adrenal glands. The addition of 10 per cent alcohol makes the extract more stable and does not interfere with its parenteral use. The solution is sterilized by Berkefeld filtration.

The complete process for 100 kilos of gland, giving a typical distribution of extractive solids and hormone activity, is outlined in the accompanying diagram. The final solution contains less than 1 part in 400,000 of epinephrine as measured by the dog blood pressure method. Most of the extracts assayed between 1:

800,000 and 1:1,000,000 of epinephrine. The extractive solids per cc. of solution representing 40 gm. of fresh glands have varied between 0.6 and 1.0 mg. Nitrogen determination by micro-Kjeldahl procedure indicates approximately 3.5 per cent nitrogen in the extracted solids.

No toxic effects have been observed following the injection of doses of extract amounting to 5 to 15 cc. per kilo subcutaneously or intravenously in guinea pigs and rabbits. The extract has been found to be suitable for human clinical study without danger of toxic reactions.

The aqueous solution containing 10 per cent alcohol is stable for at least 1 year in the refrigerator at about 4°. Assays after

TABLE I
Assay of Adrenal Cortical Extracts

Extract No.	Extractives per kilo fresh gland	Dog assay		Rat assay	
		Per kilo fresh gland	Per mg. extractives	Per kilo fresh gland	Per mg. extractives
	mg.	units	units	units	units
102-MHK-2	10.0	1500	150	83	8.3
13C-MHK-2	19.2	2500	130	104	5.4
185-MHK-2	24.5			125	5.1
[230-MHK-2	20.0	2550	127	83	4.2
245-MHK-2	25.3	3380	134	166	6.6
292-MHK-2	17.0	1800	106	83	4.9
14-MHK-5	16.7	2400	144	104	6.2
51-MHK-5	23.8	2300	97	125	5.2

1 year at room temperature indicate about 75 per cent of original activity by both rat and dog methods.

In Table I we have given the results of rat and dog assays on some typical extracts prepared by the method described. The final hormone solution contains approximately 100 dog units or approximately 4 rat units per cc., representing 40 gm. of fresh gland. This corresponds to approximately 2500 dog units per kilo of gland, which is a yield equal to those reported by Pfiffner and coworkers (11) for their better extracts. Since the extracted solids usually run slightly less than 1 mg. per cc., this gives a potency of 100 dog units per mg., which is a degree of purity greater than we have been able to obtain by the application of

the Swingle and Piffner method (3). The extract prepared by our abbreviated method would appear to offer excellent starting material for further attempts at purification of the cortical hormone. This work is being continued.

We wish to express our appreciation to Dr. David Klein and The Wilson Laboratories for their assistance in supplying the adrenal glands for these studies.

SUMMARY

A method is described for preparing adrenal extracts containing the cortical hormone. The procedure consists essentially in extracting adrenal glands with neutral acetone, removing the acetone *in vacuo*, which leaves an aqueous residue, extracting the aqueous residue with petroleum ether to remove inert fatty substances, and extracting the cortical hormone from the aqueous residue with ethylene dichloride. The residue remaining after removal of ethylene dichloride is partitioned between dilute alcohol and petroleum ether to remove a small amount of residual cholesterol. To the aqueous solution remaining after concentration of the dilute alcohol phase is added sodium chloride to make 0.9 per cent, an inactive precipitate removed by centrifuging, and the resultant aqueous solution sterilized by Berkefeld filtration.

By this comparatively simple procedure, extracts assaying 2500 dog units per kilo of fresh gland extracted and 100 dog units per mg. of extracted solids can be easily obtained. The extracts so obtained are substantially free of epinephrine and suitable for clinical study.

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THE EFFECT OF DEXTROSE INGESTION ON THE CHOLESTEROL FRACTIONS OF THE BLOOD*

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Under the above title Fitz and Bruger (1) recently published evidence which indicated that the ingestion of dextrose by man is frequently accompanied by an increase in the total cholesterol concentration of the blood serum. The experimental procedure consisted in giving 100 gm. of dextrose in approximately 15 per cent solution to fasting subjects in two equal doses $\frac{1}{2}$ hour apart. Blood samples were taken for cholesterol analysis just before the first 50 gm. dose of dextrose, $\frac{1}{2}$ hour later (just before the second dose), and $\frac{1}{2}$ hour after the second dose. The free cholesterol concentration did not change significantly in most of the experiments and, therefore, since the amount of total cholesterol increased, there was an increase in ratio of combined to free cholesterol which in some instances was marked. The average increase in the two samples of serum taken after dextrose administration was between 16 and 31 per cent in seven experiments, and even larger increases, up to 51 per cent (Subject 10, $\frac{1}{2}$ hour after first dose of dextrose), were obtained in individual samples.

This observation is surprising in view of the accumulation in this laboratory of considerable evidence indicating strongly that the proportion between the cholesterol fractions is a physiological constant (except in the neonatal period (2)), varying within a narrow range, from which deviations do not occur except under pathological conditions. In a series of 126 samples of blood serum from 91 healthy adult human subjects the minimum ratio of combined to free cholesterol was 2.32 and the maximum was

* This investigation was made possible by the support of the Josiah Macy, Jr., Foundation.

3.12 (3).¹ In over 1500 samples of serum from healthy and diseased human subjects values outside this range were observed with few exceptions only in the neonatal period and in the presence of liver disease or infection (usually acute). Particularly striking was the frequent finding of normal ratios of combined to free cholesterol in patients with marked hypercholesterolemia.

The proportion between the cholesterol fractions is not altered significantly after ordinary meals. This was shown (3) in several experiments in which the total and free cholesterol concentrations of the serum were determined before and at frequent intervals after a breakfast which in some instances was abnormally rich in cholesterol. More convincing was the finding of almost the same average percentage of free in total cholesterol and range of variation in 63 samples of serum taken during fasting and in an equal number taken after eating.

The foregoing evidence supports the conception that the proportion between the cholesterol fractions is a physiological constant which is not easily influenced by external factors. It is difficult to correlate this conception with Fitz and Bruger's claim that large changes in the ratio may be brought about by the relatively mild stimulus of ingesting dextrose. If the proportion is as labile as this result indicates, it is not easy to understand why larger variations have not been observed in this laboratory, especially in the measurements which were made during absorption.

Because of these considerations it appeared necessary to repeat the experiments of Fitz and Bruger. Their finding was not confirmed.

EXPERIMENTAL

The procedure was identical with that employed by Fitz and Bruger except for the use of the method of Schoenheimer and Sperry (4) in its original form for the determination of cholesterol. Fitz and Bruger employed Fitz's application of this method to the colorimeter (5). Care was taken to give the glucose in 15 per cent solution (Fitz and Bruger used "approximately 15 per

¹ In terms of percentage of free in total cholesterol these values are 30.1 and 24.3 per cent, respectively. The two expressions are related by the equation $P = 100/(1 + R)$, where P is the percentage of free in total cholesterol and R is the ratio of combined to free cholesterol.

TABLE I

Concentration of Cholesterol Fractions in Blood Serum before and after Administration of Dextrose

Subject No.	Specimen	Total cholesterol		Average deviation	Free cholesterol	Combined cholesterol	Ratio, combined to free cholesterol	Deviation of ratio	Average deviation
		Concentration	Deviation						
	min.	mg. per 100 cc.	per cent		mg. per 100 cc.	mg. per 100 cc.		per cent	
1	Control	182.5			45.3	137.2	3.03		
	30	173.3	-5.0		45.0	128.3	2.85	-5.9	
	60	178.0	-2.5	-3.8	43.6	134.4	3.08	+1.7	-2.1
2	Control	337.3			91.9	245.4	2.67		
	30	322.3	-4.4		91.3	231.0	2.53	-5.2	
	60	334.0	-1.0	-2.7	90.1	243.9	2.71	+1.5	-1.9
3	Control	219.0			57.1	161.9	2.84		
	30	193.8	-11.5		52.3	141.5	2.71	-4.6	
	60	206.8	-5.6	-8.6	55.5	151.3	2.73	-3.9	-4.3
4	Control	143.0			39.1	103.9	2.66		
	30	142.3	-0.5		39.6	102.7	2.59	-2.6	
	60	142.5	-0.3	-0.4	39.4	103.1	2.62	-1.5	-2.1
5	Control	223.3			62.4	160.9	2.58		
	30	223.3	0		63.9	159.4	2.49	-3.5	
	60	223.0	-0.1	-0.1	63.8	159.2	2.50	-3.1	-3.3
6	Control	275.8			75.9	199.9	2.63		
	30	301.0	+9.1		80.0	221.0	2.76	+4.9	
	60	277.0	+0.4	+4.8	79.5	197.5	2.48	-5.7	-0.4
7	Control	207.0			54.5	152.5	2.80		
	30	201.1	-2.9		53.2	147.9	2.78	-0.7	
	60	197.4	-4.6	-3.8	50.7	146.7	2.89	+3.2	+1.3
8	Control	221.8			60.2	161.6	2.68		
	30	220.0	-0.8		60.7	159.3	2.62	-2.2	
	60	223.5	+0.8	0	61.3	162.2	2.65	-1.1	-1.7
9	Control	249.5			69.2	180.3	2.61		
	30	256.8	+2.9		71.6	185.2	2.59	-0.8	
	60	264.7	+6.1	+4.5	75.4	189.3	2.51	-3.8	-2.3
10	Control	160.4			43.0	117.4	2.73		
	30	161.7	+0.8		43.7	118.0	2.70	-1.1	
	60	145.3	-9.4	-4.3	39.2	106.1	2.71	-0.7	-0.9
11	Control	184.8			46.3	138.5	2.99		
	30	168.8	-8.7		42.8	126.0	2.94	-1.7	
	60	182.7	-1.1	-4.9	45.8	136.9	2.99	0	-0.9

cent") as it was thought that the large volume of water (667 cc.) might have exerted some effect. The subjects were young, healthy, male adults.

DISCUSSION

It is evident from the data presented in Table I that no marked changes either in total cholesterol concentration or in ratio of combined to free cholesterol followed the ingestion of dextrose. Instead of increases, such as were frequently observed by Fitz and Bruger, small decreases were observed in most of the experiments, but no significance is attached to this result since the changes were in most instances little beyond or within the limit of error of the analysis. This is particularly true of the ratio of combined to free cholesterol. Although small fluctuations occurred, all values were within the limits of normal variation. In a few instances significant changes in total cholesterol concentration occurred (maximum 11.5 per cent) but equally large variations in both directions were noted following breakfast and in a few instances during fasting (3). Such apparent changes may represent in part errors in analysis and perhaps in part variations in the hydration of the blood, which might be anticipated, particularly under the conditions of the present experiments.

In seeking an explanation for the difference between the result of this investigation and that of Fitz and Bruger a rather striking discrepancy in their control analyses on fasting subjects was noted. Such control (zero time) determinations were carried out on twenty-eight persons, of whom twenty served as subjects for the experiments described above, the serum cholesterol concentration being determined 30 and 60 minutes after the fasting analysis and first dose of dextrose. In the remaining eight subjects the serum was also analyzed for cholesterol 30 and 60 minutes after the first determination but without the administration of dextrose; *i.e.*, three fasting analyses were carried out at half hour intervals. All of the analyses in five of these subjects gave values for the ratio of combined to free cholesterol within the normal range observed by the author. In the remaining three subjects all of the ratios, except one, were over 2.0; *i.e.*, only a little below the minimum found in healthy subjects (3). The results of the fasting analyses in the twenty subjects who took dextrose stand in marked contrast. In only two instances was the ratio within the author's normal range, while in eleven it was below 2.0. In six cases values between 1.6 and 1.8 were recorded.

Such low ratios have been seen in this laboratory only in the presence of liver disease or acute infection.

Although Fitz and Bruger do not comment on the marked discrepancy between the fasting results obtained in their control subjects, who received no dextrose, and in the subjects who received dextrose, it is evident that their principal finding is related to the difference, since in most instances the increase in ratio which they emphasize, following dextrose ingestion, represents changes from an abnormal (according to the author's experience) level either into or toward the normal range. On the assumption that Fitz and Bruger studied healthy subjects, as was the case in the present investigation, the discrepancy in regard to the fasting analyses (and presumably in regard to the effect of dextrose) could only be accounted for by a difference in analytical technique.

The foregoing results and discussion were submitted to Dr. Fitz and Dr. Bruger in the hope that another explanation might be found. Such was the case. They stated in a personal communication that, "We were working with old, female, bedridden patients many of whom exhibited a certain amount of cachexia and other conditions such as infectious arthritis, carcinoma, hepatic cirrhosis, etc." Low ratios of combined to free cholesterol, such as they obtained in many of their control analyses, would be expected, according to the author's experience, in the presence of such conditions. It is evidently not possible to compare the result of the present study with that of Fitz and Bruger, since the subjects were quite different. It can only be concluded that in normal persons no significant change in the proportion between the cholesterol fractions of the blood serum follows the ingestion of dextrose under the conditions of the foregoing experiments. On the other hand, in highly abnormal patients, such as those studied by Fitz and Bruger, changes in the proportion apparently may occur under the same conditions.

SUMMARY

The concentration of total and free cholesterol in the blood serum was determined before and after the administration of dextrose in healthy human subjects according to the technique of Fitz and Bruger (1). In contrast with their finding, no sig-

nificant change in the proportion between the cholesterol fractions occurred. The result offers further evidence for the constancy of the proportion under physiological conditions.

Discussion of the apparent discrepancy with Dr. Fitz and Dr. Bruger revealed that they had employed highly abnormal patients as subjects; hence it is not possible to compare the results of the two investigations.

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THE CHEMICAL ACTIVATION OF STEROLS

I. THE NATURE OF THE FLORIDIN ACTIVATION OF CHOLESTEROL*

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At the time the investigations herein described were begun little was known concerning the chemistry of the antirachitic activation of ergosterol by irradiation. An approach to the study of the chemical nature of antirachitic substances was made possible when Bills (1) reported an antirachitic catalysis of cholesterol with the fullers' earth, floridin. Although it was concluded from investigations (2, 3) at that time that the antirachitic substance thus produced was not vitamin D, it seemed that more interest should have been accorded an apparent chemical antirachitic synthesis as distinguished from the well known production of vitamin D by light rays.

When it was found that this activated substance produced an animal response to calcification and fecal pH changes which could not be distinguished from that of vitamin D, chemical studies were undertaken to clarify the mechanism of the activation of cholesterol by floridin. Some of these results were reported in a preliminary announcement (4).

Floridin is one of a number of native acid clays containing mostly hydrated silica, considerable ferric oxide, calcium, magnesium, and aluminum and a trace of some acids. Bills found maximum reactivity with cholesterol to be induced in floridin by expelling much of its water content. Bills and McDonald (2) found that the acid clay then became a good dehydrating agent which in carbon tetrachloride solution formed dicholesteryl ether as an initial substance in a chain of transformations of cholesterol leading to an antirachitic product.

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Action of Floridin on Purified Cholesterol—In order to eliminate substances activatable by light rays from interference in the interpretation of the results, the cholesterol used in the experiments here reported was treated vigorously with potassium permanganate in accordance with the procedure of Kon, Daniels, and Steenbock (3), who found that cholesterol thus treated could not be activated by ultraviolet irradiation. The Bills procedure¹ for the floridin antirachitic catalysis of cholesterol, when applied to the cholesterol thus purified, still produced the characteristic color change through red to purple and black which he described. The product when fed to rachitic rats was antirachitic, as indicated by the Zucker and Matzner (5) fecal pH test and the McCollum, Simmonds, Shipley, and Park procedure (6) for the line test.²

Constituents of Floridin Causing Antirachitic Catalysis of Cholesterol—In order to determine what ingredient of the fullers' earth brought about the transformation of cholesterol, various salts found to be present were heated to the activating temperature (280°) and their capacities to bring about the characteristic color change in carbon tetrachloride solutions of cholesterol were noted. The activated floridin gave persistent tests for small amounts of the sulfate radical. Aluminum compounds dehydrated at 280° were inactive, except aluminum sulfate heated for a short time. This salt decomposes at 280° into sulfur trioxide and aluminum

¹ The procedure essentially as described is as follows: Floridin was activated by heating 2 hours at $280^{\circ} \pm 5^{\circ}$. 1.25 gm. of cholesterol in 37.5 cc. of carbon tetrachloride were refluxed for 5 hours with 6.25 gm. of the activated floridin. The black suspension was filtered and the floridin residue extracted with ether and acetone until no more colored material passed into solution. The combined filtrate and extracts were tested on animals.

² Zucker and Matzner (5) found that the high intestinal alkalinity of rachitic rats was decreased by curative amounts of vitamin D in the ration and proposed a fecal pH test for this vitamin. The limitations of this test have been presented by Heller and Caskey (7). Its usefulness in the work here reported was limited to two interpretations of animal response, positive or negative, and relative potencies were based largely on minimum amounts of antirachitic supplement which still lowered the pH. Interpretation of the data was simplified by consideration only of the quinhydrone voltages, a consistent change to positive values constituting a positive animal response.

oxide. When heated excessively, it became inert. In other words, when sufficient sulfur trioxide was left adsorbed on the aluminum oxide, all of the color play of activated floridin on cholesterol was obtained. Therefore, it appeared as if sulfuric acid were the activating constituent of floridin.

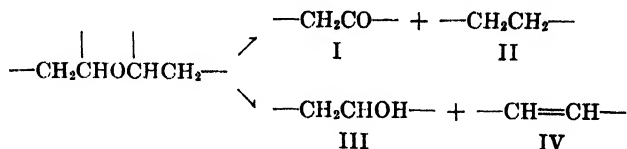
Experiments were then undertaken to determine whether an activating agent like floridin could be made with mixtures of pure silicic acid and sulfuric acid. Two such mixtures were prepared, dehydrated, and tested for this antirachitic catalysis of cholesterol as follows:

Preparation A—10 gm. of 200 mesh c.p. silicic acid, 30 cc. of water, and 3 cc. of concentrated sulfuric acid were mixed and dried at 100°, then heated rapidly to 280°. After 2 hours of heating at this temperature no more fumes of sulfur trioxide were evolved. 6 gm. of this colorless powder refluxed for 2 hours with 1 gm. of cholesterol in 30 cc. of carbon tetrachloride produced the characteristic color change and the formation of an antirachitic substance such that 14 cc. of the tetrachloride solution, evaporated on 100 gm. of the rachitogenic ration, gave a positive pH test for antirachitic activity. This procedure, when the same silicic acid was unacidified and heated, gave no color change and no antirachitic activity with cholesterol as indicated by the pH test.

Preparation B—A solution of 100 gm. of sodium silicate in 500 cc. of water was acidified with sulfuric acid, and the precipitated gel, washed by decantation until free from sulfates, was then mixed with 10 cc. of concentrated sulfuric acid and dried at 100°. The hard residue was finely ground and heated at 300° for 2 hours, then cooled, washed with water, dried, and heated to 280° for 0.5 hour. 5 gm. of this colorless powdered silica gel, when refluxed for 2.75 hours with 1 gm. of cholesterol in 30 cc. of carbon tetrachloride, gave the characteristic color change and formed an antirachitic substance. When an aliquot of the tetrachloride solution representing 250 mg. of catalyzed cholesterol was evaporated on 100 gm. of the rachitogenic ration, the animal response was positive to the pH test. Cholesterol, similarly treated with dehydrated floridin and fed at a somewhat higher level, gave a negative test for antirachitic activity. Therefore, by the use of this silica gel in place of floridin in a boiling carbon tetrachloride solution of cholesterol it was possible to produce an antirachitic

substance which, when fed at lower levels, would bring about a positive response in rachitic rats.

Nature of Transformation Products of Dicholesteryl Ether—Bills and McDonald (2) had already shown that dicholesteryl ether when refluxed with the active floridin in a solution of carbon tetrachloride produced an antirachitic substance. Since sulfuric acid is an essential reagent in the formation of an antirachitic substance from cholesterol and dicholesteryl ether, the chief action involved is one of dehydration, the first step being the removal of a molecule of water from 2 molecules of cholesterol to form 1 molecule of dicholesteryl ether. Further dehydration of the ether could occur only by the rupture of the ether linkage to make the oxygen available for the formation of more water. That the ether linkage is actually unstable when treated with floridin is shown by the fact that Bills and McDonald (2) were able to prepare amyl cholesteryl ether from dicholesteryl ether and amyl alcohol. Dicholesteryl ether could conceivably break up at the ether linkage to form the characteristic groups in cholestenone (I) and cholestene (II) or cholesterol (III) and cholesterilene (IV).



If the cholestenone-cholestene type of rupture were involved, it should be possible to activate either or both of these derivatives antirachitically by the silicic acid-sulfuric acid activating agent. For this purpose cholestenone was prepared by heating cholesterol with copper oxide according to the method of Diels and Abderhalden (8). Cholestenone, 0.5 gm., was refluxed for 1.5 hours with 2.5 gm. of the Preparation A of heated sulfuric acid-treated silicic acid in 18 cc. of carbon tetrachloride. A similar treatment of cholesterol produced the antirachitic substance, yet with cholestenone there was a negative animal response to the pH test. The characteristic color change was not produced by cholestenone.

These results pointed to a possibility of a breaking up of the ether into cholesterol and cholesterilene. If the decomposition of the ether takes place in this manner, it should be possible to

demonstrate the presence of cholesterol among the adsorbed products of the floridin used in a brief catalysis of pure dicholesteryl ether. Cholesterilene, if produced in the decomposition, could possibly be hydrolyzed to cholesterol as reported by Fantl and Kabos (9) and in accord with the recognized action of concentrated sulfuric acid upon compounds containing an ethylenic double bond.

Accordingly 1.5 gm. of dicholesteryl ether were refluxed in 70 cc. of carbon tetrachloride with 6.25 gm. of dehydrated floridin for 15 minutes. The mixture was filtered, and the floridin residue washed once with carbon tetrachloride and then extracted with anhydrous ether. From this ether extract, as well as from the above carbon tetrachloride filtrate, dicholesteryl ether was precipitated upon the addition of absolute methyl alcohol. The alcoholic filtrate was evaporated to a small volume and, on cooling, plates separated which, after precipitation from the alcoholic solution with a little water and recrystallization from alcohol, melted at 149–150°. This product mixed with a sample of commercial cholesterol, which had been recrystallized from acetone, melted at 148–149°. The cholesterol-cholesterilene type of rupture of the ether linkage was thereby substantiated.

Antirachitic Catalysis of Cholesterilene with Sulfur Trioxide—Further proof of such a transformation was obtained when it was found that cholesterilene, prepared from cholesterol by the copper sulfate method of Mauthner and Suida (10), could be made antirachitic by refluxing in carbon tetrachloride solution with the heated acidified silica gel (Preparation B). On the other hand unheated sulfuric acid in ignited silicic acid would not develop antirachitic activity with cholesterilene. This furnished more evidence that sulfur trioxide was necessary for this antirachitic catalysis. In fact it was then found that 2 moles of sulfur trioxide in carbon tetrachloride formed an antirachitic substance from cholesterilene such that 10 mg. produced positive pH and line tests in rachitic rats.

Sulfonation in Antirachitic Activation of Cholesterol—The antirachitic activation of cholesterilene by sulfur trioxide in carbon tetrachloride resembled the Courtot (11) procedure for the sulfonation of aromatic compounds so closely that the main reaction in the transformation was considered as a sulfonation of chole-

terilene. Friese (12) had developed a satisfactory method for the sulfonation of hydroaromatic compounds, particularly of cyclohexene, and produced cyclohexanol-*o*-sulfonic acid in good yield by the graded action of sulfuric acid and acetic anhydride in acetic acid solution. After considerable experimentation and modification this sulfuric acid-acetic anhydride sulfonation was applied with a measure of success to cholesterolene.

The procedure ultimately adopted was as follows: To a mixture of 3.68 gm. (0.01 mole) of cholesterolene and 36 cc. of glacial acetic acid were added with stirring 2.1 gm. (0.02 mole) of 95 per cent sulfuric acid. Next 4.0 cc. (0.04 mole) of acetic anhydride were admixed and the colored solution heated with the container closed to moisture for 4 hours on a boiling water bath. Water was then added and the acetic acid evaporated on the water bath. Water was again added to the residue and the remaining acetic acid evaporated. The final residue was soluble in about 100 parts of water.

An aliquot of this solution, representing 2.2 mg. of cholesterolene, when evaporated on the rachitogenic ration and consumed by each rachitic rat, in 7 days produced a positive pH test and a broken or narrow continuous line of calcification. When 7.5 mg. were consumed per rat over a 14 day period, the pH test was positive and calcification practically complete. These rats consumed feed normally and showed no noticeable abnormalities upon postmortem examination.

Since cholesterolene is practically water-insoluble and the reaction product was water-soluble, tests were undertaken to determine the nature of the sulfuric acid addition which had taken place to cause this change. Cholesteryl hydrogen sulfate is hydrolyzable in boiling dilute acid and not in dilute alkali according to Gardner and Fox (13). The reaction product was stable in boiling dilute hydrochloric acid as well as in boiling dilute alkali.

Essentially the same sulfonation procedure as was applied to cholesterolene was applied next to cholesterol to determine whether the reaction could be a step in the floridin antirachitic catalysis. It was found that the treated equivalent of 9 to 12 mg. of cholesterol consumed per rat in 9 days produced a positive pH test.

Separation of Sulfonic Acid Salts—The foregoing experiments seemed to indicate that the sulfonation of cholesterolene was in-

volved in the chemical transformation of cholestérol into an anti-rachitic substance. Sulfonic acids if formed probably would be somewhat soluble in water and difficultly hydrolyzable. Also they should form rather insoluble alkaline earth salts because of the large molecular weight of the hydrocarbon. It was found that barium, calcium, or magnesium ions when added to a water solution of the reaction product precipitated copiously large floccules of insoluble salt.

The complete procedure ultimately adopted for the separation of the barium salt was as follows: The water solution of the acid residue obtained from 3.68 gm. of cholesterilenę by the sulfonation process, as above described, was treated with 0.1 N barium acetate until no further precipitation occurred. The precipitate was collected on a suction filter, washed by stirring with water and by suction filtering. The precipitate, dried in a vacuum and weighing about 5 gm., was digested in two 50 cc. portions of hot 95 per cent alcohol, filtered by suction, and dried in a vacuum. The crude salt was then mixed with 25 to 30 cc. of carbon tetrachloride and dissolved by the dropwise addition of absolute alcohol with stirring. The solution was filtered through a covered fluted filter. The rapid admixture of 100 cc. of absolute alcohol precipitated the barium salt which was collected on a suction filter and dried in a vacuum. Three preparations of the salt by this procedure gave the following analysis.

$(C_{27}H_{45}O_3S)_2Ba$. Calculated.....		Ba 13.30, S 6.21
Found.....		" 13.18, " 6.28
		" 13.21, " 6.16
		" 13.38, " 5.94

This salt does not noticeably decompose or fuse below 330° and is obtained as described in straw-colored microscopic granules. The free acid is quite soluble in water and makes a soapy solution from which on evaporation the acid separates as a stiff oil. The nature of the synthesis of this acid, its stability to hydrolysis, its solubility, and its salt-forming property and the analytical data all characterize this sulfuric acid addition product as a sulfonic acid.

The free sulfonic acid was liberated from three preparations of the barium salt and tested for antirachitic potency. It was found

that 3 to 4 mg. of the acid consumed per rat in 8 days produced positive pH tests and approximately narrow continuous lines of calcification.

The calcium salt was prepared by the same procedure modified only by the substitution of calcium hydroxide for barium acetate and acetone for alcohol. This salt forms straw-colored granules when anhydrous which fuse at 320–325°. It forms a red wax with a small amount of water. A sample of the anhydrous salt gave the following results on analysis.

(C ₂₇ H ₄₅ O ₅ S) ₂ Ca.	Calculated...	Ca 4.29, S 6.86
	Found.....	" 4.37, " 6.68

This sample of the calcium salt gave a positive pH test and a broken line of calcification when 3 mg. were consumed per rat during 8 days.

The procedure for activation of cholesterol by this modified sulfonation process and for the separation of the calcium salt yields with cholesterol regularly from 60 to 70 per cent of the theoretical of a salt of a sulfonic acid. Four such preparations gave the calcium salt with an average percentage of calcium approximating the theoretical for this salt of the sulfonic acid.

Preparation No.	112	113	115	119	Average
Ca.....	4.30	4.29	4.14	4.35	4.27

DISCUSSION

Since no relatively great variation in potency was evident in the reaction products from the numerous modifications in procedure, the data presented do not indicate the formation of a highly potent product such as can be produced from ergosterol by irradiation. Antirachitic potency was in general increased in accordance with the effectiveness of sulfonation and the purified sulfonic acid derivative of cholesterol was the most antirachitic. Fractional precipitation from carbon tetrachloride solution with alcohol did not decrease the potency of the derivative. Different preparations of the free sulfonic acid appeared to have about the same potency.

Since Friese found that the addition of sulfuric acid to cyclohexene occurred under relatively mild conditions to produce cyclohexanol-*o*-sulfonic acid, one would expect cholesterol by

analogy to be sulfonated in a similar manner. If the sulfonation of cholesterilene were strictly analogous, a sulfonic acid would be formed containing 1 molecule of water more than the analytical data of the present investigations indicate. Another view of the sulfonation process set forth by Michael and Weiner (14) would question the addition of sulfuric acid in the sulfonation of alkenes

in the form given by Friese, thus $\text{—CHOH—CHSO}_3\text{H}$, and support an "aldolization" addition, such as —CH=CSO(OH)_2 ,

convertible to $\text{—CH=CSO}_3\text{H} + \text{H}_2\text{O}$. If sulfur trioxide activates cholesterilene through sulfonation, the aldolization mechanism only would be tenable in the activation according to the theory of Michael and Weiner.

There is a possibility of a number of isomers and a shifting of double bonds in the nucleus of cholesterilene during the process of sulfonation. Since there are two double bonds, either of these might be sites for sulfonation. However, the sulfonation has been considered as occurring at the site of the double bond created by the removal of a molecule of water from cholesterol for the following reasons: (1) cholestenone could not be activated by the same procedure which activated cholesterol, (2) dicholesteryl ether broke up into cholesterol and apparently cholesterilene before activation, and (3) preferential sulfonation at a double bond on a dinuclear carbon atom is rare if not unknown. Until more data on this subject are available, the reaction product which produced the positive antirachitic animal response should be known by the most general term, cholesterilene sulfonic acid.

SUMMARY

1. Sulfur trioxide or sulfuric acid heated to evolve sulfur trioxide was found to be a constituent of floridin capable of activating cholesterol antirachitically.

2. The conversion of dicholesteryl ether into cholesterilene was found to be a possible step in this antirachitic transformation of cholesterol.

3. Cholesterilene made by another method was antirachitically activated (a) in carbon tetrachloride solution by sulfur trioxide or

heated sulfuric acid in silicic acid and (b) in acetic acid solution by sulfuric acid in the presence of acetic anhydride.

4. The method for antirachitic activation (3, a) was recognized as a process of sulfonation and the main reaction product of the best activating procedure (3, b) with cholesterolene was isolated and identified as the barium and calcium salts of a mono-sulfonic acid of cholesterolene.

5. The sulfonic acid liberated from the barium salt was antirachitic.

6. The calcium salt of cholesterolene sulfonic acid was antirachitic.

7. The procedure employed in the activation and sulfonation of cholesterolene also produces a sulfonic acid and a similar calcium salt from cholesterol.

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A CHEMICAL STUDY OF THE BLOOD OF ALLIGATOR MISSISSIPPIENSIS

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In the course of an investigation concerning perfusion of the alligator heart (Rosenblatt, 1936) it was found desirable to have data available on the normal constituents of the blood of this animal. The only data reported are those of Austin, Sunderman, and Camack (1927) whose work was concerned with the serum electrolyte changes in the alligator with variations in body temperature. Dill and Edwards (1931) have reported an investigation of the physicochemical properties of the blood of the crocodile (*Crocodilus acutus*, Cuvier).

On the whole, little detailed information is available concerning the chemical composition of the blood of the lower vertebrates. Furthermore, since the alligator is, in essence, somewhat of a connecting link between the cold blooded vertebrates, on the one hand, and the higher warm blooded animals, on the other, it was thought that an investigation of the constituents of the blood of this animal would be of interest.

Methods

The studies were all conducted on eight young specimens of the species, *Alligator mississippiensis*, whose size varied from 24 to 30 inches. The animals were kept immersed in constantly changing water at temperatures varying from 20–24°. The experiments were performed in the spring. This point is of great significance, as will be indicated later in the paper.

Blood was obtained directly from the heart and was either allowed to clot or was heparinized for whole blood and blood plasma protein determinations. The following analyses were

performed on the blood samples: sodium by the Butler and Tut-hill (1931) modification of the Barber and Kolthoff method (1928); potassium by the Kramer and Tisdall (1921) method with Kerr's (1926) modification of the trichloroacetic acid filtrate; calcium by the Kramer and Tisdall method (1921); inorganic phosphorus by the Kuttner and Cohen (1927) method; chlorides by the Eisenman (1929) modification of the open Carius method; CO₂-combining power by the Van Slyke and Cullen method (1917); non-protein nitrogen by the colorimetric method of Folin and Wu (1919). Total proteins were determined by the Howe (1921) micro-Kjeldahl method; fibrinogen, albumin, and globulin by the micro-Kjeldahl method of Cullen and Van Slyke (1920). The refractive index was determined on plasma at room temperature with the Abbe refractometer and calculated according to Reiss (1904) as follows:

$$\frac{(I_s - I_w - 0.0028)}{0.00172} = \text{gm. of protein per 100 cc.}$$

where I_s is the refractive index of the plasma; I_w , the refractive index of water; 0.0028, the arbitrary correction for salts and non-electrolytes.

DISCUSSION

The results of the investigation are recorded in Tables I and II. As will be evident from the data, there was great variation in different animals. This variation may be explained in part by the fact that the animals were investigated at the beginning of their so called feeding period. It is known that the alligator will take food in the latter part of spring and will continue to eat until September; the remainder of the year is passed in hibernation without food intake. It is possible that the nutritional state of the animals may be the cause of the variations in the concentration of plasma electrolytes and non-electrolytes.

On the other hand, the plasma protein in all except Animal 8 was quite consistent. This is extremely interesting since Animals 3, 4, and 5 were never fed, whereas Animals 6, 7, and 8 were given ample food. The total protein content, however, showed no great variation, although the plasma of Animal 8 contained considerably more protein than the others.

TABLE I
Electrolytes and Non-Protein Nitrogen Distribution between Cells and Plasma of Alligator Blood

Animal No.....	1	2	3	4	5	6	7	8
Sodium								
Whole blood, m.-eq. per l.							105.06	95.3
Plasma, m.-eq. per l.	148.6	139.8	121.5	137.6	134.9	137.4	134.6	132.6
Cells " "							28.6	19.7
Potassium								
Whole blood, m.-eq. per l.						20.2	21.6	28.1
Plasma, m.-eq. per l.		7.1	5.8	5.6	6.3	4.4	6.2	9.6
Cells " "						83.3	61.2	65.4
Ca, serum, " per l.								
" " mg. per 100 cc.		6.0	6.1	6.2	6.5			
Inorganic P, serum, mg. per 100 cc.		12.0	12.2	12.4	12.9			
Plasma chloride, m.-eq. per l.			6.5	8.8	11.0			
Plasma CO ₂ -combining power, vol. %	98.2	99.4	81.3	92.4	83.2	86.8	87.0	80.4
Non-protein N		44.3	35.7	18.0	28.0	31.0	28.4	48.5
Whole blood, mg. per 100 cc.			76.8	73.8	66.8	82.8	84.4	112.0
Plasma, mg. per 100 cc.	24.6	22.0	47.3	34.7	28.9	29.6	30.6	31.0
Cells " " 100 "			195.0	197.5	168.6	295.6	222.8	276.4
Hematocrit, %			20	24	27	20	28	33
Refractive index, plasma	1.3460	1.3452	1.3465	1.3468	1.3453	1.3467	1.3458	1.3492

Although the fibrinogen content of alligator blood is the same as that of warm blooded animals, the albumin-globulin ratio of the plasma was found to be completely reversed from the conditions found in most of the warm blooded vertebrates. There is a much greater concentration of globulin than albumin. This finding was consistent in all animals, both fasted and fed, and it seems that this reversed albumin-globulin ratio is a normal characteristic of the alligator blood. Demenier (1934) has reported such a ratio in the serum of various fishes and it is in the realm of possibility that other cold blooded vertebrates may also reveal such an albumin-globulin ratio.

On the whole, the protein content of the alligator blood plasma

TABLE II
Blood Plasma Proteins (in Gm. per 100 Cc. of Plasma) of Alligator

Animal No	1	2	3	4	5	6	7	8
Refractive index	1.3460	1.3452	1.3465	1.3468	1.3453	1.3467	1.3458	1.3492
Total protein (as calculated from refractive index) . .	5.7	5.29	5.7	6.0	5.3	6.3	5.3	7.5
Total protein (micro-Kjeldahl)			5.48	5.63	5.37	5.98	5.16	7.24
Fibrinogen				0.43	0.37	0.41	0.295	0.40
Albumin			1.39	0.81	1.74	1.35	1.02	1.62
Globulin			4.09*	4.48	3.26	4.22	3.85	5.22
A:G ratio			0.34	0.18	0.53	0.32	0.27	0.29

* Includes fibrinogen.

is slightly lower than that of warm blooded animals. This is in agreement with Dill and Edwards' (1931) finding in the crocodile and Austin, Sunderman, and Camack's data on the alligator (1927).

In Table I are recorded the results obtained for the electrolyte and non-protein constituents of the blood. The sodium and potassium concentrations of the plasma are slightly lower than the figures recorded by Dill and Edwards (1931) on the crocodile. In Animals 6, 7, and 8 whole blood and plasma analyses were conducted to determine cell content of sodium and potassium. The data obtained indicate that alligator blood contains more potassium in the cell than sodium, and the reverse is true of the

plasma. Evidently the distribution of sodium and potassium between the cells and plasma in the blood of the alligator is similar to that in most mammals.

The calcium concentration in the plasma was quite constant, being, however, slightly higher than in mammals. In the light of Roe and Dyer's (1934) finding of a high calcium concentration in the chick, this result in the alligator may be of evolutionary significance.

The serum inorganic phosphorus was found to be much higher than in warm blooded animals. The figures recorded for the alligator are in agreement with McCay's (1931) work on the phosphorus content of the turtle blood serum. McCay has shown that animals which have nucleated blood cells have a higher concentration of phosphorus than animals with non-nucleated red cells.

The chloride concentration in the plasma was found to be much lower than the figures given by Dill and Edwards (1931) for the crocodile and Austin, Sunderman, and Camack (1927) for the alligator. It is possible that the low chloride recorded in this paper may be the result of the nutritional state of the animal and the season of the year.

The carbon dioxide-combining power, in most animals, varied from 18 to 35 volumes per cent. In Animals 2 and 8 values of 44 and 48 volumes per cent were found. Again, one must keep in mind that these animals were being observed at a time of the year when there is a great instability.

One of the most interesting results obtained was in connection with the non-protein nitrogen of the cells and plasma. The concentration of non-protein nitrogen in the plasma is for the most part in the limits of variation of warm blooded animals, but in all cases there was found an extremely high concentration of non-protein nitrogen in the cells. It is possible that the high cellular non-protein nitrogen may be the result of a higher metabolism of the alligator red blood cell. Vars (1934) reports high non-protein nitrogen values for the fish and for the turtle. Gonzaga (1936) has found high non-protein nitrogen during the early life of the chick, with a subsequent decrease as the age increased. It is in the realm of possibility that these high figures of the alligator red blood cell are of evolutionary significance, since in the

lower vertebrates, especially the elasmobranchs, there is an extremely high non-protein nitrogen concentration and in the warm blooded vertebrates there is a great reduction, with the alligator falling into an intermediary position.

SUMMARY

Data are presented on the sodium, potassium, calcium, chloride, CO₂-combining power, inorganic phosphorus, non-protein nitrogen, total protein, fibrinogen, albumin, and globulin of alligator blood. The most interesting results are a reversed albumin-globulin ratio of the blood plasma proteins and a high cellular non-protein nitrogen. The significance of these findings is discussed from a phylogenetic point of view.

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THE CHEMICAL IDENTITY OF CERTAIN BASIC CONSTITUENTS PRESENT IN THE SECRETIONS OF VARIOUS SPECIES OF TOADS

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In a previous paper (1) we described the isolation of certain basic compounds, as flavianates, from the secretions of different species of toads and pointed out that some of these substances form series of identical compounds. We also reported that the secretions of certain species of toads contain basic constituents which possess a pharmacological action similar to that of epinephrine,¹ and we expressed the opinion that these substances are derivatives of tryptamine. The subsequent work of Wieland and his associates (6) proved that this inference was correct. These workers established the chemical structures of these active principles by synthesis and showed that they could be divided into two closely related chemical compounds: (a) bufotenine, 5-hydroxy-3- β -dimethylaminoethylindole, and (b) bufotenidine, the betaine of bufotenine.

In this paper we wish to report our findings concerning the more definite characterization of some of the flavianates previously described. For this purpose these compounds were converted into other salts which would allow a more precise identification.

The physiologically active flavianates obtained from ch'an su and the secretions of *Bufo bufo gargarizans*, *Bufo fowleri*, and *Bufo formosus* yielded picrates and hydriodide salts identical with the corresponding salts of bufotenidine. These salts showed similar melting points and gave no depression in melting points when mixed. The chemical analysis of each of the various salts also indicates that they are identical (Tables I and II).

¹ This base has been found to be present in ch'an su (2) and in the secretions of *Bufo marinus* (3), *Bufo arenarum* (4, 5), and *Bufo regularis* (4).

The flavianates obtained by us from the secretions of *Bufo bufo bufo* (*Bufo vulgaris*) and *Bufo viridis viridis* that were found to be similar to the above flavianates in physiological action gave picrates that seem to be identical with the picrate of bufotenine, as described by Wieland and coworkers (6). The chemical anal-

TABLE I

Bufotenidine Picrate

Calculated for $C_{13}H_{18}ON_2 \cdot C_6H_3O_7N_3$. C 51.01, H 4.70, N 15.67.

Source	M.p.	Analysis		
		C	H	N
	°C.			
Ch'an su.....	198	50.72	4.84	15.33
<i>Bufo bufo gargarizans</i>	198	50.88	4.94	15.46
<i>Bufo fowleri</i>	198	51.12	5.01	15.39
<i>Bufo formosus</i>	197	50.82	4.95	15.60

The salt crystallized from ethyl alcohol in red needles. Mixed melting points showed no depression.

TABLE II

Bufotenidine Hydriodide

Calculated for $C_{13}H_{18}ON_2 \cdot HI$. C 45.09, H 5.50, N 8.08.

Source	M.p.	Analysis		
		C	H	N
	°C.			
Ch'an su.....	209	44.92	5.65	7.92
<i>Bufo bufo gargarizans</i>	209	45.21	5.72	7.98
<i>Bufo fowleri</i>	209	44.88	5.69	8.00
<i>Bufo formosus</i>	209	45.02	5.22	8.12

The salt crystallized from methyl alcohol in prisms. Mixed melting points showed no depression.

yses also support the assumption of the identity of these salts with that of bufotenine (Table III).

Along with these physiologically active principles, a certain other basic constituent has been obtained as a flavianate (melting at 265–270°) from ch'an su and the secretions of *Bufo marinus* and *Bufo arenarum*; this compound, however, is physiologically inactive. It is identical with the product obtained on hydrolysis

of bufothionine, a sulfur-containing substance present in the secretions of certain toads (4, 7) (Table IV). This observation agrees with the view of Wieland and coworkers (6) in regard to the chemical identity of these basic constituents.

TABLE III
Bufotenine Picrate

Calculated for $C_{12}H_{16}ON_2 \cdot C_6H_5O_7N_3$. C 50.00, H 4.39, N 16.20.

Source	M.p.	Analysis		
		C	H	N
	°C.			
<i>Bufo bufo bufo (Bufo vulgaris)</i>	177	49.84	4.61	15.94
<i>Bufo viridis viridis</i>	178	49.85	4.51	15.91

The salt crystallized from methyl alcohol-ether in red needles. Mixed melting points showed no depression.

TABLE IV
Picrate of Base, $C_{12}H_{14}ON_2$

Calculated for $C_{12}H_{14}ON_2 \cdot C_6H_5O_7N_3$. C 50.11, H 3.95, N 16.24.

Source	M.p.	Analysis		
		C	H	N
	°C.			
Ch'an su.....	183-184	50.15	4.12	15.95
<i>Bufo marinus</i>	183-184	49.98	4.22	16.01
<i>Bufo arenarum</i>	183-184	49.85	4.08	15.89
Bufothionine.....	183-184	50.02	4.18	15.92

The picrate crystallized from water in long yellow needles that contained 1 molecule of water of crystallization, which is given off at 100° in a vacuum. The analytical data given in the table are those found for the anhydrous salt. Mixed melting points of the various picrates showed no depression.

At present we have no definite information in respect to the exact chemical structure of this base, $C_{12}H_{14}ON_2$. We have found that its molecule contains one methylimide group; methoxyl groups are not present. On the basis of certain color reactions, we believe that, like bufotenine and bufotenidine, it is a derivative of indole.

EXPERIMENTAL

The different flavianates were converted into the various salts following the method of Wieland and his coworkers (6). Most of the analyses reported in this paper were carried out by Dr. Ing. A. Schoeller, Berlin-Schmargendorf. The analytical data given represent the average value of two or more determinations.

The flavianate of the base $C_{12}H_{14}ON_2$, melting at $265-266^\circ$, isolated from the secretion of *Bufo marinus* was converted into the free base and certain salts, which were analyzed. The free base was obtained on addition of ammonia to an aqueous solution of the acetate. It crystallized from alcohol in rhombohedrons which begin to darken at 200° and melt at 240° with decomposition; the crystals gradually darken when left in the open air (oxidation).

<i>Analysis</i> — $C_{12}H_{14}ON_2$.	Calculated.	C 71.29, H 6.93, N 13.86
	Found	" 71.04, " 7.12, " 13.62

The following salts of the base $C_{12}H_{14}ON_2$ from *Bufo marinus* were prepared in the usual manner and found to have properties similar to those described by Wieland and coworkers (7) for the base obtained on hydrolysis of bufotohionine.

The hydrochloride crystallized from alcohol-ether in needles, which begin to decompose at 212° , melt at 240° , and lose 1 molecule of water when dried *in vacuo* at 90° .

<i>Analysis</i> — $C_{12}H_{14}ON_2 \cdot HCl$.
Calculated. C 60.37, H 6.29, N 11.74, Cl 14.88, NCH_3 12.16
Found. " 59.88, " 6.18, " 11.51, " 14.56, " 12.10

The acid sulfate crystallized from alcohol-ether in rosette-like crystals, melting at 205° .

<i>Analysis</i> — $C_{12}H_{14}ON_2 \cdot H_2SO_4$.	Calculated, N 9.33; found, N 8.98
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The acetate crystallized from alcohol-ether in prisms, which begin to decompose at 210° and melt at 215° .

<i>Analysis</i> —
$C_{12}H_{14}ON_2 \cdot CH_3COOH$. Calculated, NCH_3 11.10; found, NCH_3 12.15

The hydriodide crystallized from alcohol-ether in rhomb-shaped crystals, which begin to darken at 220° and melt at 238° .

Analysis—

$C_{12}H_{14}ON_2 \cdot HI$.	Calculated.	C 43.64,	H 4.55,	N 8.49,	NCH ₃ 8.79
	Found.	" 43.42,	" 4.50,	" 7.95,	" 9.22

Hydrolysis of Bufothionine—35 mg. of bufothionine obtained from the secretion of *Bufo arenarum* (4) were boiled for 5 minutes with 1 cc. of 2 N HCl. The solution was evaporated *in vacuo* and converted into the picrate. The picrate crystallized from water in long needles, which showed a melting point of 183–184° and which showed no depression in melting point when mixed with the picrate of the base $C_{12}H_{14}ON_2$ from *Bufo marinus*.

SUMMARY

Bufotenidine has been shown to be present in ch'an su and in the secretions of *Bufo bufo gargarizans*, *Bufo fowleri*, and *Bufo formosus*. Bufotenine has been obtained from the secretions of *Bufo bufo bufo* (*Bufo vulgaris*) and *Bufo viridis viridis*. A basic constituent having the composition $C_{12}H_{14}ON_2$ was found in ch'an su and in the secretions of *Bufo marinus* and *Bufo arenarum*. This substance is identical with the compound that one obtains on hydrolysis of bufothionine, a sulfur-containing constituent present in the secretion of certain species of toads.

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STUDIES ON THE COPPER AND IRON CONTENT OF TISSUES AND ORGANS IN NUTRITIONAL ANEMIA*

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Although the necessity of copper for hemoglobin formation in several species has been well established (discussed by Elvehjem (1)), nothing is known about the chemical nature of the physiological effects of copper. Progress in this direction has been slow because extremely small amounts of copper are involved and because it is not definitely known whether the effects of copper are of a general systemic nature or whether they are localized to the organs and tissues concerned with hematopoiesis.

There is evidence that copper may concentrate in centers of hematogenic activity. It has been reported (2, 3) that during the development of the chick embryo, the copper of the egg becomes concentrated in it, particularly so at the time when pulmonary respiration starts and when there is very rapid hemoglobin formation. Ramage *et al.* (4) have called attention to the high concentration of copper in the human liver at birth. In pig embryos practically all of the copper of the embryo is concentrated in the liver during the early stages of development; *i.e.*, at a time when this organ is most active hematopoietically (5). Bence (6) has also pointed out the relatively high copper content of embryonic livers of humans and calves. Little information is available regarding the copper content of bone marrow. Sarata (7) found from 2 to 3.6 mg. of Cu per kilo of fresh red marrow of rabbits. In chronic hemorrhagic anemia the copper content was increased to about 5 mg. per kilo. Fatty bone marrow, however,

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contained only 0.7 to 0.9 mg. of Cu per kilo. These figures were interpreted to indicate that "when blood formation is accelerated copper accumulates in the bone marrow" and that "it is reasonable to conclude that the copper participates actually in the formation of blood." In a case of human aplastic anemia the red marrow was found to contain 5 mg. per kilo of fresh tissue, the fatty marrow 4.6 mg. per kilo (6). Tompsett (8) found that the copper content of human ribs showed great variation, while that of the corresponding vertebræ was constant. This might be due to differences in the amount of active bone marrow present in the ribs or to variations in the copper content of it.

Considering these facts it is not unreasonable to expect that an animal with severe nutritional anemia due to iron and copper deficiency should contain only very small amounts of copper. Should this animal be fed copper, or iron and copper, for short periods of time it might be expected that the copper would be concentrated in those tissues where it is needed for hematopoiesis and where this process is most active after the initiation of iron and copper feeding. Only very small amounts of copper are necessary to bring about hematopoiesis in the anemic rat having access to available iron (9, 10); 0.01 mg. of Cu daily gives a rapid response and even smaller amounts are slowly effective. Only very sensitive methods of analysis can permit a successful attack of such a problem. With a sensitive, fairly rapid, and reliable method for copper analysis available, the present study was undertaken in an attempt to determine, if possible, the site of action of copper in the formation of hemoglobin.

EXPERIMENTAL

Analytical Methods

With the exception of the ribs, all tissues analyzed were ashed with sulfuric and perchloric acids. After ashing was complete, the digest was treated with 2 cc. of 2 per cent purified Na_2SO_3 and boiled to decompose any excess perchloric acid that might be present. The digest was then neutralized with concentrated NH_4OH with paranitrophenol as an indicator. Aliquots were analyzed for copper and iron. The bony tissues were ashed in platinum dishes in an electric furnace at incipient red heat.

The white ash was dissolved in 1:1 HCl. Suitable aliquots were used for the copper and iron analyses.

Copper Analysis—In all this work we have used the method of Fischer and Leopoldi (11). Its great advantage lies in the fact that no preliminary separation of copper is required and that it is accurate and sensitive. The details of our procedure have recently been reported by Coulson (12). The method gives good results with amounts of copper as low as 4 micrograms and with very careful work even 3 micrograms. When dealing with such

TABLE I
Recovery of Cu (in Micrograms) Added to Biological Material

	Cu in material	Cu added	Cu found	Cu recovered
Ash solution from 0.73 gm. bone	3.76 3.56	4	7.64	3.98
" " " 0.91 " "	4.44 4.11	4	8.50	4.22
2 gm. liver; 10 micrograms Cu added before ashing; $\frac{1}{3}$ aliquots analyzed	7.80 7.60		9.74 9.44	1.89

TABLE II
Copper Content of Blood (in Micrograms) Determined by Direct Analysis and by Calculation from Copper Content of Plasma and Blood Cells

Fig No.	Cell volume	Cu per 100 cc. blood		Cu in plasma	Cu in blood cells
		Calculated	Found		
12	36.6	113.8	108.3	85.3	28.5
11	7.29	12.1	11.1	8.3	3.8
13	8.1	10.4	9.7	7.3	3.1

small amounts it is necessary to use only 1 cc. of the dithizone reagent and to extract for about 10 minutes. Also the volume of CCl_4 used for washing purposes must be reduced so that the final volume will not exceed 4 cc. This method has given very good results in our hands, as indicated in Tables I and II. Wet ashing with perchloric and sulfuric acids does not lead to loss of copper, as claimed by Sarata (13).

Total Iron—Aliquots of the ash solutions obtained in the manner previously described were analyzed by the bipyridine method of

Lintzel (14). The bipyridine reagent used was dissolved in 10 per cent acetic acid; the hydroquinone was added as a solid. We have used this method for biological materials for about 3 years (15) with much success, particularly with materials high in calcium and phosphorus.

Inorganic Iron—The method used for inorganic iron analysis was essentially that described by Kohler *et al.* (16). Because the acidity of the solution in which the iron-bipyridine complex is formed should not be greater than pH 2.5 and because quantitative extraction of the inorganic iron from the tissues should be made at a pH not higher than 5, the concentration of trichloroacetic acid used was varied with the size of the sample. In the case of livers of anemic pigs a concentration of 2.5 per cent trichloroacetic

TABLE III
Copper Content (in Micrograms) of Rats

Rats	No. of animals	Liver		Carcass, total Cu
		Total Cu	Cu per gm. dry liver	
Normal, 6 wks. old	4	19.4		192.6
Severely anemic	13	2.7	4.1	41.3
Getting 0.5 mg. Fe daily for 7 days	6	2.7	3.2	42.3
“ 0.1 “ Cu “ “ 7 “	12	7.9	9.2	61.8
“ 0.5 “ Fe + 0.1 mg. Cu daily for 7 days	7	10	9.2	71.2

acid in 40 per cent ethyl alcohol was used. When working with ribs, 5 per cent trichloroacetic acid was used on account of the buffering capacity of the calcium phosphates of the bone. In most cases the acidity of the solutions used for colorimetry was checked with the quinhydrone electrode and found to be in the desired range.

Work with Rats—All animals used in this work were young rats made severely anemic on an exclusive milk diet. When the hemoglobin content of the blood was about 2 gm. per 100 cc., part of the litter was killed by decapitation and taken for analysis. Litter mates were fed 0.5 mg. of purified iron daily as FeCl_3 or 0.1 mg. of Cu as CuSO_4 daily, or both, for a period of 7 days. This period is sufficient to raise the hemoglobin content of an

anemic rat getting both iron and copper from 2 gm. to 8 to 10 gm. of hemoglobin per 100 cc. of blood. The hematopoietic organs, therefore, should have been, at the time of killing, in a state of maximum activity. Copper determinations were made by the method indicated. Because preliminary experiments indicated that the copper content of spleen, heart, kidneys, and brain was too small to permit separate analysis, these organs were included in most experiments in the "carcass," *i.e.*, the body minus the digestive tract, part of the blood, and the liver. Table III summarizes the results obtained in this manner.

These animals consumed on an average only about 25 cc. of milk per day, which contains 3 to 4 micrograms of Cu. Allowing for the whole period a total Cu intake from the milk of 30 micrograms and 700 micrograms from copper sulfate, the copper retention of these animals (average of 26 micrograms) during this period was only $26/730 = 3.5$ per cent for those getting copper only and $37/730 = 5$ per cent for those getting both iron and copper (average retention of 37 micrograms). This is particularly remarkable in view of the fact that the hemoglobin response of the animals getting both iron and copper was maximal. (The fact that the intestines were not included in the analyses does not affect these figures seriously, since there are only about 3 micrograms of copper in the intestinal organs of animals of this age on a milk diet (17).) The figures given for the copper content of our severely anemic rats are somewhat lower than 52 micrograms quoted by Lindow *et al.* (17) and 51 micrograms by Bing and co-workers (18). This is probably due to the fact that our animals were more severely anemic and, therefore, more depleted of their copper stores. Very low copper retention during rapid hemoglobin regeneration by anemic rats after feeding of iron and copper has also been noted by Bing *et al.* (18), who record a retention of 12 per cent over a period of 17 days when feeding 0.5 mg. of Fe and 0.025 mg. of Cu daily.

These results are indeed surprising when it is considered that the animals were extremely depleted of copper and that they were unable to regenerate hemoglobin upon iron therapy alone. The argument that copper was poorly absorbed is hardly valid because maximum hemoglobin regeneration was observed. It is more plausible to assume that most of the copper was actually

absorbed and reexcreted after exerting its action. It is entirely possible that the copper which was actually retained became concentrated in the hematopoietic centers, the bone marrow, and perhaps the spleen (19) and the liver. Indeed some retention of copper by the liver has been observed. The remainder of the retained copper (20 to 30 micrograms) could conceivably have been deposited in the bone marrow.

Since we are dealing with such small quantities of copper and since the organs of anemic rats are so small, these studies could not be successfully continued with this species. Larger animals had to be used which yielded enough material to permit analysis of all organs that might be involved in blood formation. Because sufficient material was available for analysis, we included in this study some determinations of the total and inorganic iron content of the different organs.

*Work with Pigs*¹—Young pigs were chosen as the experimental animals because the nutritional anemia produced in this species on a milk diet seems to be in all respects comparable to that of the rat (20, 21) and since pigs also grow and develop rapidly to maturity on a diet of mineralized milk (22, 23).

During the first 5 weeks of their life, the pigs had been kept on wooden floors and clean shavings and they were denied access to the ration consumed by the mother. After that they were kept either singly or in pairs in wooden boxes and thus rigorously denied all access to metals except those contained in the diet. This consisted of cow's milk exclusively, which was fed in earthen jars several times daily. The boxes were washed carefully every day. On such a regimen the animals more or less rapidly developed a severe anemia, as shown by hemoglobin determinations by the Newcomer method. Control animals were kept on a milk diet fortified with 25 mg. of Fe + 2 mg. of Cu daily. When the pigs were severely anemic they were either killed or fed purified iron, copper, or a combination of both, before being sacrificed (see Fig. 1).

Viviperfusion was used to kill the animals because it was desired to collect sufficient blood for analysis and to free the tissues as much as possible from blood so that the analytical results

¹ We are indebted to Professor J. M. Fargo of the Department of Animal Husbandry for the pigs used in our work.

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would not be complicated by the iron and copper content of the blood. For this purpose glass cannulae were inserted under ether anesthesia into an external jugular vein and into a carotid artery. Fresh glucose-Locke's solution, kept at body temperature and saturated with oxygen, was run under pressure into the jugular vein and blood was withdrawn at the same time from the carotid, the first samples being used for analysis. At intervals plethora was produced by stopping the carotid outflow in an attempt to achieve more complete perfusion of the tissues. Under these

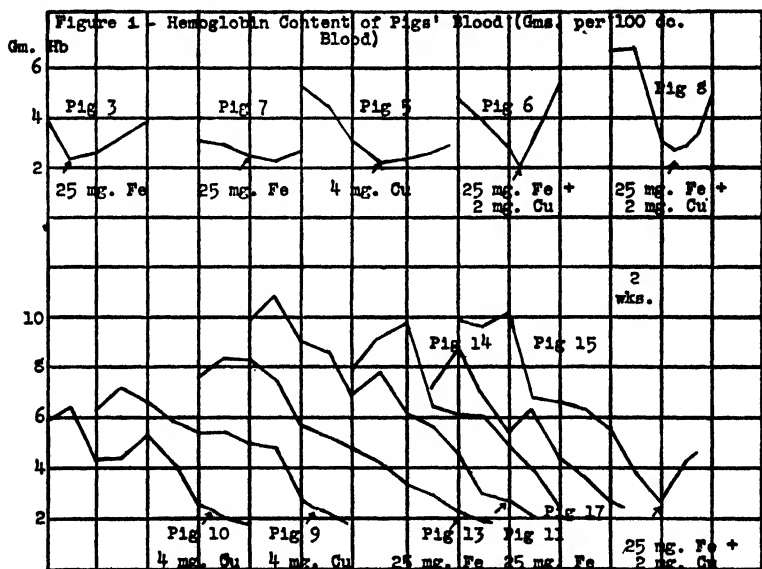


FIG. 1. Hemoglobin content of pig blood. The arrows indicate when feeding of the daily supplement was started.

conditions the heart of the animals survived usually until the out-flowing liquid was practically colorless. After heart action ceased, perfusion was continued until 4 to 6 liters of solution were used. Then the tissues desired for analysis were removed, care being taken to avoid contamination.

The efficiency of the method is illustrated best by the fact that during the last stages of perfusion the liquid flowing from the cut end of the animal's tail was colorless. Very recently Hahn and Whipple (24) have commented on the efficiency of viviper-

fusion in dogs. No claim is made that all tissues are completely washed free from blood by our method. Perfusion of the bone marrow and the spleen was perhaps rather incomplete. Attention has recently been called to the difficulty of perfusing spleens (25). Owing to anatomical peculiarities the pig's spleen seems to be specially hard to perfuse.

It was hoped that from the long bones of the pigs enough bone marrow could be collected to permit analyses for iron and copper. In these young animals, however, the cavities of the long bones were so densely filled with a trabecular network that no red marrow could be obtained. The distal ends of the ribs (about 1 inch) were therefore chosen to represent bone marrow because they are the least calcified portion of the ribs and contain proportionately more red marrow than the middle section. Hahn and Whipple (24) have resorted to a similar procedure, using the whole rib as representing bone marrow. They consider that one-third of the weight of the rib is parenchymatous tissue. The moisture content of bone marrow is undoubtedly very variable, although high in the gelatinous type of marrow contained in ribs. Assuming an average moisture content of the red marrow of 80 per cent and considering the rib to consist of one-third red marrow, the copper content of rabbit marrow quoted by Sarata (7) was 15 mg. per kilo of dry marrow and that found here for normal pigs was 22 mg. per kilo. These figures fall roughly into the same range. Taking only the distal end of the rib for analysis is justified by the fact that the copper content of the middle portion of the ribs, containing less red marrow, was only from 22 to 48 per cent of that found in the distal portion.

The tissues removed were dried in an electric oven at 100-105° and stored for analyses. Some of the tissue, however, was used fresh for the determination of inorganic iron and of moisture.

DISCUSSION

The analytical results obtained are presented in Table IV. They are complicated by the fact that not all animals were from the same litter. Of a litter of eight pigs farrowed in March, 1935 (Pigs 1 to 8), three were severely anemic at the time of weaning and died before they were really put on the experiment. It was impossible to perfuse them. It is interesting to note that the

copper content of their livers was comparatively high, while the total iron content, although the animals were not perfused, was rather low. This would suggest that these animals suffered from anemia primarily due to lack of iron. Lintzel (26) has already called attention to the very low iron content of livers of new born

TABLE IV
Organ Analysis (Pigs)

The results for the organs are expressed in micrograms per gm. of dry organ.

Pig No.	Treatment	Hb per 100 cc. blood	Liver			Spleen			Distal end of rib		
			Total Fe	Inorganic Fe	Cu	Total Fe	Inorganic Fe	Cu	Total Fe	Inorganic Fe	Cu
		gm.									
1.	Died from anemia		57.5		74.9	770.0		7.3	46.6		5.0
2	Same		109.8		102.5	426.0		11.9	44.5	6.1	6.7
3	"	2.60	98.0		42.1	408.0		6.1	73.2		4.3
14	Anemic; no metals	2.50	69.6	17.4	7.4	198.0	69.3	2.9	48.4	5.3	5.4
17	Same	2.40	74.7	27.4	5.6	468.0	66.6	3.7	50.7	5.9	2.6
3	Fe only	3.94	71.5		17.9	637.0		5.2	67.9	24.1	4.7
7	Same	2.55	87.4		10.2	649.0		3.1	94.2	27.0	4.1
11	"	2	124.3	33.8	5.5	605.0	75.1	2.8	128.0	15.2	9.1
13	"	2	97.0	31.9	6.0	525.0	87.4	2.6	69.5	9.6	2.4
5	Cu only	2.87	62.5	18.8	81.4	369.0	40.6	6.3	35.4	12.3	6.3
9	Same	2	237.0	41.5	25.2	413.0	57.2	8.1	60.5	9.0	6.4
10	"	2	193.7	21.0	19.2	410.0	150.0	9.9	53.4	11.5	7.9
6	Fe + Cu 7 days	5.40	67.9		26.4	434.0		5.1	92.7	33.9	5.3
8	" + " 9 "	4.85	60.6		24.1	475.0		5.6	85.5	19.0	4.9
15	Same	4.51	67.7	21.5	9.7	492.0	57.1	5.6	64.8	6.8	4.4
12	Fe + Cu; normal control	9.95	68.0	30.7	13.7	292.0	83.0	7.0	78.0	8.1	10.5
18	Same	10.11	59.6	21.0	13.3	351.5	87.3	5.4	39.3	5.2	4.6

pigs. Copper, in agreement with Bence (6), was found to be stored in the liver of the young animal in such amounts as to constitute a reserve on which the young can draw during the early period of low copper intake. During this period treatment with iron alone should bring about a temporary improvement in

the blood picture as it has been observed in the case of Pig 3 and by others (20, 27). Those animals that survive this early crisis deplete their copper stores and eventually suffer from both iron and copper deficiency. Hamilton *et al.* (21) have pointed out that anemia in pigs is not only more prevalent in early spring, but also attended by greater mortality than in summer and fall, although the pigs are reared under identical conditions. This work substantiates their observations, because pigs from two litters farrowed in the middle of April (Pigs 9 and 10) and late in

TABLE V

Average Results of Analyses of Organs (Pigs)

The results are expressed in micrograms per gm. of dry organ.

Treatment	Liver			Spleen			Distal ends of ribs		
	Total Fe	Inorganic Fe	Cu	Total Fe	Inorganic Fe	Cu	Total Fe	Inorganic Fe	Cu
Died from Fe deficiency; not perfused	88.1		73.2	533		8.4	54.3		5.3
Anemic; Fe and Cu deficiency	72.2	22.4	6.5	333	68.0	3.1	49.5	5.6	4.0
Fe only for 14 days	95.0	32.9	9.9	604	81.2	3.4	89.9	19.0	5.1
Cu " " 14 "	62.5*	27.1	41.9	397	82.6	8.1	49.8	10.9	6.9
Fe + Cu " 7 to 9 days	65.4	21.5	16.7	461	57.1	5.4	81.0	20.2	4.9
" + " daily; normal controls	63.8	25.8	13.5	322	85.2	6.2	58.7	6.7	7.6

* The abnormally high figures for total iron of Pigs 9 and 10 are not included.

August (Pigs 11 to 18), respectively, were much more resistant to the onset of nutritional anemia. When they did finally attain a state of severe anemia, the copper content of their livers was much lower than that of the animals dying from anemia in early spring. The iron content of the livers, however, was about the same in both groups.

The results of the tissue analyses presented in Table IV are summarized in Table V. From them the following facts become apparent.

1. During the first few weeks of life young pigs possess a con-

siderable storage of copper in the liver. This, however, becomes rapidly depleted if the animals are restricted to a diet very low in iron and copper, and an anemia due to lack of both these metals will result.

2. Feeding 25 mg. of pure iron daily for 1 to 2 weeks to severely anemic pigs causes a small increase of both inorganic and total iron in the liver, spleen, and ribs. This increase is most pronounced in the spleen and the ribs. Very little of the iron thus deposited is in the form of inorganic iron.

3. Feeding 4 mg. of copper daily alone for 14 days increases the copper content of the liver to some extent. Small increases in the copper content of the spleen and distal ends of the ribs are also observed.

4. When both iron and copper are fed to a severely anemic pig for a short time, a small increase in the copper content of liver and spleen, but not of the distal ends of the ribs (bone marrow), is observed. Evidently there is no marked accumulation of copper in these tissues under these conditions. An increase in the total and inorganic iron content of the distal ends of the ribs can be noted, however.

5. Normal young pigs receiving 25 mg. of iron and 2 mg. of copper daily do not show appreciable accumulation of these elements in the tissues. The amounts fed, apparently, are not sufficient to permit storage.

It is interesting to compare the analyses for total iron with those reported by Hahn and Whipple (24) for the perfused tissues of a dog exhausted of iron reserves. Assuming the water content of the dog's organs to be 78 per cent for liver and 81.5 per cent for spleen (these figures represent the average values for the pig organs of this work), the iron content of the dog's liver is calculated as 77 micrograms per gm. of dry tissue, the iron content of the spleen as 420 micrograms per gm. of dry tissue. These figures are in the same range (although a little higher) than those observed in the case of severely anemic pigs suffering from iron and copper deficiency.

It must be emphasized that there is considerable variation among the results obtained from animals receiving the same treatment. This is due to the fact that not all pigs used were litter mates, were not born at the same time of the year, and re-

quired varying periods of time before they were depleted of their stores of iron and copper available for hemoglobin formation. The average results presented in Table V merely indicate the trend of the changes in iron and copper content of liver, spleen, and distal ends of the ribs (bone marrow). A comparison of the tissue analyses of litter mates (Figs 1 to 8 and 11 to 18, respectively) gives a more absolute picture. Very surprising and not conforming to expectations is the high iron content of the livers of Figs 9 and 10. These animals were severely anemic when feeding of copper was initiated. There was no evidence of hemoglobin or erythrocyte regeneration. These two animals were born the middle of April, 1935, and were free from contact with iron and copper, as were all others. They developed anemia very slowly, like the pigs born 4 months later, which showed much lower iron content of the livers. Pigs 9 and 10 died and could not be perfused, although in one case much blood was drained from the liver. As compared to 215 micrograms per gm. of dry liver found in Figs 9 and 10, the iron content of blood containing 2 gm. of hemoglobin per 100 cc. is about 335 micrograms per gm. of dry blood. It is unlikely that the very high iron content of these livers could be accounted for solely by the blood retained by the tissue.

From the results of the tissue analyses it is evident that there was no great accumulation of copper in the liver, spleen, and the distal ends of the ribs of anemic animals getting iron and copper for short periods of time. Hemoglobin and erythrocyte formation proceeded rapidly in these animals. Evidently it is impossible to localize the action of copper in the sense that failure of the anemic animal to regenerate is due to the fact that the copper concentration of any of the three organs mentioned is too low. That the distal ends of the ribs (bone marrow), the apparent seat of hemoglobin and erythrocyte formation, revealed these results refutes the hypothesis implied by Sarata (13) that accumulation of copper in the bone marrow cells is necessary for blood formation.

SUMMARY

1. Severely anemic rats are markedly depleted of bodily copper stores. In spite of this, feeding of iron and copper for 7 days

causes only a small retention of copper (5 per cent), although maximum hemoglobin formation is observed.

2. Young pigs may suffer from anemia due to iron deficiency only. In the presence of bodily stores of copper this anemia responds to treatment with iron only.

3. Young pigs suffering from both iron and copper deficiency cannot form hemoglobin and erythrocytes when pure iron alone is supplied.

4. Feeding of iron and copper for a short period of time to severely anemic pigs suffering from iron and copper deficiency does not lead to an accumulation of copper in the distal ends of the ribs (bone marrow), although hematopoiesis proceeds rapidly.

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STUDIES ON THE COPPER CONTENT OF THE BLOOD IN NUTRITIONAL ANEMIA*

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The occurrence of copper in the blood of man and various animals has been known for a long time but until recently most of the analyses for blood copper were made incidental to other studies. The recorded observations on this subject have been summarized in the papers by Locke *et al.* (1), Sarata (2), Roncato and Bassani (3), and Tompsett (4). With the discovery of the essential nature of copper for hemoglobin formation (5) the analysis of animal organs and tissues for copper has assumed new significance and during the last few years attempts have been made to study systematically the copper content of the blood of man and animals in normal and pathological conditions. Several observations are recorded in the literature about the changes of the copper content of the blood in various conditions of anemia (1, 6-11), but, as far as we know, no data have been published on the changes in the copper content of the blood of animals suffering from nutritional anemia under well controlled conditions. Because in recovery from severe nutritional anemia the bone marrow is in a state of maximum activity, this condition should be unusually favorable for studying the copper content of the blood during rapid hematopoiesis.

Our studies on the copper content of tissues and organs of anemic pigs provided excellent material for copper analyses of the blood and made it possible to correlate the results obtained from the analyses of the various organs and tissues.

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EXPERIMENTAL

In the preceding paper (12) the methods for handling the animals, for blood collection, and for copper analysis are described. The results in Table II of that paper indicate the good agreement between the copper content of the blood obtained by direct analysis and that calculated from the copper content of the plasma, the blood cells, and from the cell volume. The blood was collected in flasks containing purified potassium oxalate as anti-coagulant. Before analysis the blood cells were washed twice with purified physiological salt solution.

The copper analyses reported in Table I were obtained from the animals used in the study reported in the preceding paper (Figs 1 to 18). The results made it desirable to follow the copper content of the blood of pigs as they developed anemia and recovered from it under the influence of iron and copper feeding. For this purpose a litter of pigs was raised and fed as in the other experiment except that small amounts of pure iron were fed to some of the animals for short periods of time in order to delay the onset of anemia. This was desirable because our previous work had indicated that pigs, particularly those born in early spring, will become severely anemic owing to iron deficiency before their bodily stores of copper are exhausted. When the animals were 5 weeks old, weekly bleedings for copper analysis were started. The blood was withdrawn under light ether anesthesia by heart puncture. The amount of blood withdrawn depended upon the expected copper content of the blood as indicated by the previous analysis. With few exceptions enough blood was withdrawn to permit copper analysis of the whole blood and of the plasma. From these values and the cell volume the approximate copper content of the blood cells could be calculated.

DISCUSSION

The results obtained from Figs 1 to 18 are summarized in Table I. The copper content of the blood obtained at a slaughterhouse from mature hogs gave values of the same magnitude as those reported by McFarlane (13), 103 to 180 micrograms of Cu per 100 cc. of blood, and by Tompsett (4) who found 165 to 185 micrograms of Cu per 100 cc. of blood and 161 to 200 micro-

grams of Cu per 100 cc. of plasma. In the case of two pigs used as control animals for these experiments, *i.e.* those on a milk diet fortified with iron and copper, the copper content of the whole blood was 108 and 133 micrograms per 100 cc.; that of the plasma 135 and 138 micrograms per 100 cc. Two control pigs subjected to weekly bleedings had the following copper distribution at the

TABLE I
Copper Content of Blood of Pigs

Pig No.	Treatment; daily supplement	Hb	Hemato- crit	Cu in 100 cc.		Cu in r.b.c. from 100 cc. blood
				Blood	Plasma	
		<i>gm. per 100 cc.</i>		<i>micro- grams</i>	<i>micro- grams</i>	<i>micro- grams</i>
14	Anemic; no metals	2.50	8.1	7.8	0.0	8.5
17	Same	2.40	6	7.3	<4	2.5
3	25 mg. Fe 14 days	3.94	17.43	98.5	58.7	50
7	Same	2.55	9.75	79.8	46.4	38
11	25 mg. Fe 8 days	2	7.29	11.1	8.9	3.8
13	25 " " 9 "	<2	8.10	9.7	7.8	3.1
5	2 " Cu 7 " 4 mg. Cu 7 more days	2.87	11.05	206	178	37.0
6	25 mg. Fe + 2 mg. Cu 7 days	5.40	17.35	231.0	218	50.9
8	25 mg. Fe + 2 mg. Cu 9 days	4.85	17.30	224.5	169.0	85.0
15	Same	4.51	14.50	120.3	129.9	18.0
12	25 mg. Fe + 2 mg. Cu 4 wks.	9.95	36.60	108.3	134.5	28.5
18	25 mg. Fe + 2 mg. Cu 24 days	10.11	28.3	133.0	137.5	21.6
Mature hogs from Madison slaughter-house				154.0	170.4	53.6
				166.0	176.2	49.0
				163.0	168.2	63.6

time of the last bleeding: 135 and 187 micrograms of Cu per 100 cc. of blood, 155 and 203 micrograms of Cu per 100 cc. of plasma, and 27 and 52 micrograms of Cu in the cells of 100 cc. of blood.

In severe nutritional anemia, however, the copper may almost disappear from the blood, the observed values being 7 and 8 micrograms per 100 cc. of whole blood and less than 4 micrograms per 100 cc. of plasma. It must be pointed out that these results

were obtained with animals suffering from both iron and copper deficiency (Pigs 14 and 17), whose livers were extremely depleted of copper. Not in all cases of severe nutritional anemia may such low values for the copper content of the blood be encountered. It was pointed out before that pigs may become severely anemic from iron deficiency alone. In that case the copper content of the blood, although reduced, does not reach the low values just mentioned. This is indicated by the values obtained with Pigs 3 and 7 which became severely anemic but responded very slowly to treatment with pure iron alone (or did not show further decrease of Hb), as shown by the hemoglobin curves in Fig. 1 of the preceding paper. The copper content of the blood of these pigs was 99 micrograms per 100 cc. of whole blood and 59 and 46 micrograms per 100 cc. of plasma. Also Pig 19, 4 weeks old, with hemoglobin less than 2 gm., had 89 micrograms of Cu per 100 cc. of blood. These facts must be strictly considered in future studies; namely, that severe nutritional anemia is not necessarily associated with extreme depletion of the copper content of the blood. Two pigs (Nos. 11 and 13), severely anemic, failed to respond to feeding of pure iron; in fact, the hemoglobin content of their blood continued to decrease. Upon analysis their blood and the livers (12) were found to be extremely low in copper. Evidently their anemic condition was due to both iron and copper deficiency. In the absence of bodily stores of copper and with a very low copper content of the blood, they were unable to form hemoglobin and erythrocytes. Analogous to this condition, only reversed, is that in which severely anemic pigs were unable to respond to treatment with copper alone (Pigs 5, 9, 10, Fig. 1 (12)). Unfortunately blood analyses from only one of these animals were obtained. The other two died suddenly during the hot and sultry days of July, 1935. From these analyses, however, it is evident that the feeding of copper raised the copper content of the blood to values higher than normal. Similarly, high values for copper were observed in pigs recovering rapidly from severe anemia under the influence of feeding iron and copper. Under the conditions of these experiments, 2 mg. of copper were supplied to the animals daily. If any of the tissues active in blood formation were in great need for copper, it should be expected that they would withdraw from the blood the copper

absorbed from the intestine. That this, apparently, is not the case to any great extent is borne out by the copper analyses of liver, spleen, and the distal ends of ribs (12).

These observations point to an intimate correlation between the copper content of the blood and the rate of hemoglobin formation. Of the tissues and organs studied in our work, the blood was the only one where a great accumulation of copper was found when copper was fed to an animal previously depleted of copper.

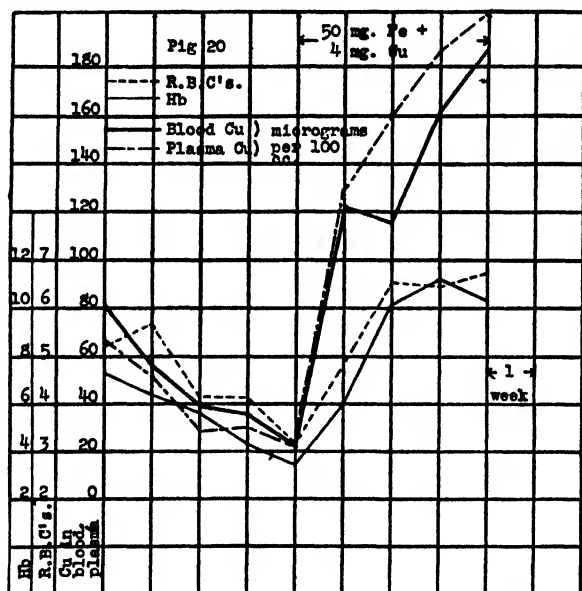


FIG. 1. Hemoglobin, erythrocyte, and copper content of the blood of Fig 20. Rapid recovery from anemia after feeding 50 mg. of Fe + 4 mg. of Cu.

This might suggest the theory that, unless the copper content of the blood is maintained at a certain minimum level, hemoglobin formation cannot take place or only at a very slow rate, even if the animal has access to available iron. To get more information on this important point weekly copper determinations of the blood of pigs were made. The results are represented in Figs. 1 to 3.

Our previous observation that in severe nutritional anemia due to iron and copper deficiency the copper content of the blood

falls to very low levels is fully substantiated. It is interesting to note that this drop in blood copper at first is very rapid, from 90 to 100 micrograms to about 30 micrograms per 100 cc. of blood. When the blood copper had reached this level, the feeding of 50 mg. of pure iron per day was started. As a result we observed a slow hemoglobin regeneration in three cases (Figs 22, 24, 25).

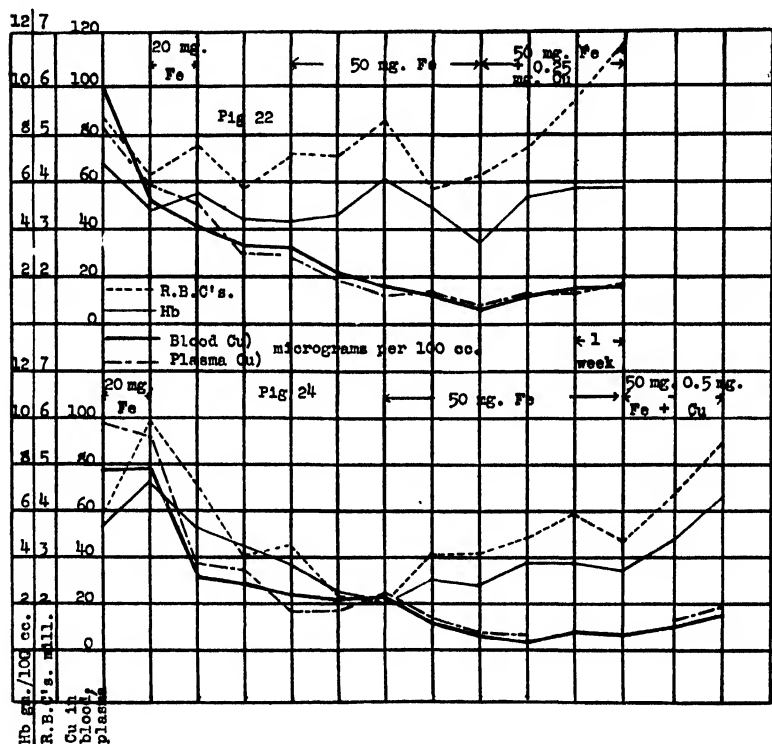


FIG. 2. Hemoglobin, erythrocyte, and copper content of the blood of Figs 22 and 24.

One animal responded more rapidly (Fig 23) for a period of 2 weeks, during which time it was able to maintain the copper content of its blood almost constant. Then, however, the copper content of the blood fell to still lower levels and hemoglobin formation in all cases became very slow or ceased, although the animals had access to an abundance of available iron. When the copper content fell to about 10 micrograms per 100 cc. of blood,

hemoglobin formation was completely arrested. It appeared, therefore, that the level of blood copper at which hemoglobin formation is possible lies somewhere between 10 and 30 micrograms per 100 cc. of blood. By feeding small amounts of copper to the pigs that had ceased to form hemoglobin, we attempted to estab-

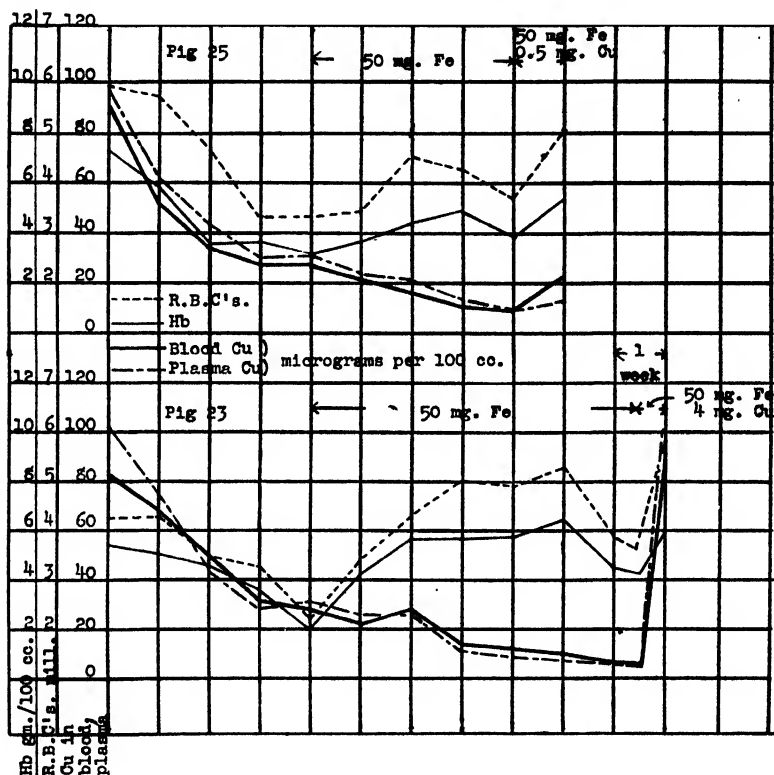


FIG. 3. Hemoglobin, erythrocyte, and copper content of the blood of Pigs 23 and 25. Note the rapid increase in blood copper after feeding 4 mg. of Cu to Pig 23.

lish at what level of blood copper hemoglobin formation would be resumed. In two cases (Pigs 22 and 24) the feeding of small amounts of copper initiated considerable hemoglobin formation, although the copper content of the blood failed to rise above 20 micrograms per 100 cc. of blood.

When larger amounts of copper were fed (4 mg. daily) we ob-

served again a tremendous increase in the copper content of the blood, together with rapid hematopoiesis. In one case (Fig 23) the copper content of the blood rose in $3\frac{1}{2}$ days from less than 10 to about 100 micrograms per 100 cc. If sufficient copper is supplied, the animal evidently attempts to restore the depleted copper content of the blood at once. Thus the accumulation of copper under these conditions is much greater in the blood than in the liver (12) and rapid hematopoiesis apparently can take place only if the copper content of the blood is at a fairly high level.

Some authors have postulated that copper is a specific stimulant for erythrocyte formation (14, 15). Following experimental hemorrhage Sarata and Suzuki (11) observed an increase in the copper content of the erythrocytes of rabbits, particularly marked during the first few days following the operation. Itizyo (16) suggested that "the cuprocytes [the copper-rich cells] are replaced by ordinary ones in a relatively short time." When we followed the copper content of the blood of our pigs during production of anemia and during recovery, the calculated copper content of the erythrocytes expressed for 1 million red blood cells per c.mm. showed a marked decrease compared to normal values. But only when large amounts of copper were fed with iron did we find the copper content of the erythrocytes increased over the values calculated for the previous bleeding. Our data can neither support nor refute the alleged production of "cuprocytes" as observed by the Japanese workers, because we worked with a different species and produced nutritional rather than hemorrhagic anemia and because we could not make copper analyses immediately upon initiation of hematopoietic activity of the bone marrow. We should like to point out, however, that it is not possible to assign to copper or to iron a specific function for either formation of erythrocytes or of hemoglobin. The two processes are interdependent and although erythrocytes may vary in their hemoglobin content, they always contain some hemoglobin. Even if hemoglobin formation proceeds only slowly, enough may be formed to permit production of large numbers of cells with a low color index. We have observed this in one of our animals (Fig 22) to which only a small amount of copper was fed.

Our observation that in nutritional anemia due to iron and

copper deficiency the copper content of the blood is so much decreased is not opposed to clinical observations that the copper content of the blood is increased in various conditions of anemia (1, 6-11) and in pregnancy (17, 18). With greater demand for formation of hemoglobin and erythrocytes the organism apparently has the ability to raise the copper content of the blood from bodily stores of copper in an effort to speed up hematopoiesis. If in addition to copper other factors such as iron and the pernicious anemia factor are available, the anemia will be overcome rapidly. If one or more of these factors is absent, hemoglobin and erythrocyte formation will not take place in spite of the high copper content of the blood and anemia will persist. What controls the regulation of the copper content of the blood is unknown. Whatever that mechanism may be, it exerts its effect rapidly (11). The observation has been repeatedly made that blood serum from animals subjected to low oxygen tension contains substances which stimulate hematopoiesis (hemopoietins) (19). This effect might perhaps be correlated with an increased copper content of the serum. Somogyi (20) observed that the stimulating effect of "regeneration serum" may be further enhanced by increasing the copper content of this serum.

From the present knowledge of the factors instrumental in hematopoiesis it is difficult to appreciate the full significance of our observations. The great increase of copper in the blood of our anemic animals following copper feeding is not only the result of increased copper absorption from the intestine. It is a well known fact that the increase in the amino acid, fat, and sugar content of the blood during the absorptive period is relatively small and transient. Increased calcium intake does not lead to a permanently increased calcium level in the blood. It might be argued that our observations are of no functional significance but that the changes in the copper content of the blood are merely secondary to and concurrent with copper storage in various organs and tissues. This, however, is unlikely, because no appreciable accumulation of copper was observed in the liver and the bone marrow (12) at a time when the copper content of the blood was high and hematopoiesis rapid. It might be suggested, however, that the developing erythrocytes in which hemoglobin is formed are bathed in and nourished by the blood. Hemoglobin

formation in and maturation of the blood cells can take place only if the surrounding environment, the blood, possesses the proper physical and chemical properties. Among such properties could be conceivably oxidation-reduction potential, sulfhydryl groups, and enzymatic activation. With low copper concentration in the blood the medium surrounding the young blood cells would not possess the required properties, and therefore, could not permit hemoglobin formation and maturation of erythrocytes.

No conclusions can be drawn as to the possible interrelation of the increased copper content and other chemical changes of the blood observed. It has been reported that after hemorrhagic anemia glycolysis (21, 22) and respiration (23) of the blood cells are increased. Attempts have been made to correlate the increased respiration with the younger forms of erythrocytes appearing in the blood during recovery (24, 25), particularly with the reticulocytes (26). It has been pointed out by several investigators that glycolysis is activated by copper (27, 28). The observation that during recovery from hemorrhagic anemia the glutathione content of the blood is increased has been recorded frequently (29-33). It is interesting to note in this connection that, in contrast to the reduction of oxidized glutathione, the oxidation of the reduced compound is not enzymatic but metallic (34). The catalytic effect of iron and particularly of copper on the oxidation of cysteine is well known (35-37). A similar situation may apply to the accelerated oxidation of reduced glutathione by blood serum (38), although it has been shown that pure glutathione *in vitro* is not oxidized by free iron or copper (39). Whether the observation (40) that the oxidation-reduction potential of the bone marrow becomes more positive in hemorrhagic anemia is related to the chemical changes observed in the blood in this condition is a matter of conjecture.

Further intensive study on the chemical changes occurring in the blood during recovery from anemia may go far in explaining the necessity of copper for hemoglobin formation and the mechanism of its action.

SUMMARY

1. In pigs suffering from nutritional anemia due to iron and copper deficiency the copper content of the blood falls to extremely low levels.

2. Feeding 2 to 4 mg. of copper per day together with iron results in a very rapid and significant increase of the copper content of the blood.

3. When small amounts of copper are fed together with iron the increase in the copper content of the blood is only small and hematopoiesis is slow.

4. It is suggested that rapid, continued hematopoiesis cannot take place unless the copper content of the blood is maintained above a minimum level. This level may be about 20 micrograms per 100 cc. of blood of pigs.

5. The significance of these observations is discussed and it is suggested that the study of the chemical changes in the blood during recovery from severe nutritional anemia offers the most promising approach toward an understanding of the function of copper in blood formation.

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THE FORMATION OF HEXOSEPHOSPHATE ESTERS IN FROG MUSCLE*

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Many of the intermediary reactions leading to the formation of lactic acid in muscle have been elucidated by work carried out in the laboratories of Meyerhof (1) and Parnas (2). There are, among others, two questions which have not been settled; namely, the point of entrance of inorganic phosphate in the glycolytic cycle and the position of hexosemonophosphate in the glycolytic scheme.

Experiments performed recently (Hegnauer and Cori (3)) suggested that hexosemonophosphate was formed by esterification of carbohydrate with inorganic phosphate. When frog muscles were kept anaerobically in Ringer's solution containing epinephrine, they showed, in comparison to control muscles kept without epinephrine, a marked rise in hexosemonophosphate and a corresponding decrease in inorganic phosphate without significant changes in phosphocreatine and adenosinetriphosphate.

It has been demonstrated that hexosediphosphate is formed by esterification of carbohydrate with the labile phosphoric acid groups of adenosinetriphosphate. If there should be a similar mechanism in the formation of hexosemonophosphate, one would expect a decrease in phosphocreatine in the experiments referred to above. Esterification with adenosinetriphosphate involves a transfer of phosphocreatine phosphate to adenylic acid in the process of resynthesis of adenosinetriphosphate (Lohmann (4)). This reaction of adenylic acid with phosphocreatine occurs with

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great rapidity in anaerobic muscle, while the resynthesis of phosphocreatine is slow and incomplete under anaerobic conditions. The phosphocreatine content is therefore an index of the participation of adenosinetriphosphate in phosphorylation, especially if the muscle is poisoned with iodoacetate, a procedure which prevents the resynthesis of phosphocreatine under anaerobic conditions.

When isolated frog muscle is subjected to a 10 second tetanus, one finds a decrease in phosphocreatine, no change in adenosinetriphosphate, and an approximately equal increase in lactic acid and hexosemonophosphate hexose (Fisher and Cori (5)). Under these conditions there is no means of deciding whether the decrease in phosphocreatine is related to the formation of lactic acid or of hexosemonophosphate. Since the phosphocreatine content remained unchanged when the hexosemonophosphate content of muscle was increased by means of epinephrine, it seemed probable that the decrease in phosphocreatine observed during stimulation of muscle was related to the formation of lactic acid (or of hexosediphosphate, since this is the precursor of lactic acid) rather than to that of hexosemonophosphate.

In order to test this assumption, it was necessary to find substances which would increase lactic acid formation without causing an appreciable increase in hexosemonophosphate. 2,4-Dinitrophenol and caffeine in small concentrations were found to answer this purpose. Furthermore, since hexosediphosphate does not accumulate in unpoisoned muscle, it was necessary to prevent its disappearance by blocking the path to lactic acid by means of iodoacetate. The problem, then, was to investigate in muscle poisoned with iodoacetate the type of hexosephosphate which would accumulate under the influence of epinephrine or dinitrophenol and to determine which phosphate compound furnished its phosphate for esterification.

EXPERIMENTAL

All experiments were carried out on the thin leg muscles of *Rana pipiens*. One group of matched muscles, obtained from two to three frogs and weighing from 1.5 to 2 gm., served as a control, while the other group was treated with various drugs. After preparation and weighing, each group of muscles was transferred to wide mouthed test-tubes containing 5 cc. of bicarbonate-

Ringer's solution. O_2 or N_2 containing 5 per cent CO_2 was bubbled through the solution, giving a pH of 7.0. The temperature was 20° throughout. The muscles were first kept in O_2 for 1 to 2 hours. They were then made anaerobic in the same Ringer's solution or in some cases after transfer to fresh Ringer's solution. After a few minutes of anaerobiosis the drugs used in the various experiments were added. At the end of the anaerobic period the tubes were quickly cooled in ice and brought to a cold room ($3-5^\circ$). The contents of the tube were transferred to a mortar and quickly ground with acid-washed sand in 5 per cent trichloroacetic acid, the final dilution being 1:10. The whole mass was filtered through an ash-free filter, and to an aliquot was added ammoniacal magnesia mixture¹ for the determination of inorganic phosphate. The rest of the filtrate was used for the determination of hexosemonophosphate (7) and of the hydrolysis curve in N HCl. Phosphocreatine was determined as the difference between directly determined and inorganic P.

Effect of Iodoacetate—Carc was taken to analyze the muscles before rigor due to iodoacetate developed because of the marked chemical changes which occur in muscle as the result of rigor. The following tests were made in order to find out how long the muscles could be kept with safety in the iodoacetate solution under anaerobic conditions. Sartorius muscles were fastened to a light lever writing on a smoked drum and were immersed in Ringer's solution containing iodoacetate in a concentration of 1:20,000. It took at least 90 minutes before the muscles began to go into rigor, although a slight degree of shortening was often noticeable after 60 minutes. This limited the time iodoacetate could be allowed to act to 60 to 90 minutes.

In 1 hour iodoacetate produced only slight changes in phosphate distribution, as shown in Table I. The phosphocreatine P was, on an average, 6 mg. per cent lower than in the controls. There was no change in the pyrophosphate fraction (difference between 0 and 8 minute hydrolysis value), no demonstrable accumulation of hexosediphosphate (difference between 8 and 30 minute

¹ We are indebted to Dr. Sacks for the composition of the magnesia mixture, which was as follows: ammonium chloride 70 gm., magnesium chloride 50 gm., aqua ammonia 200 cc. (sp. gr. 0.90), made up to 1000 cc. (Sacks and Sacks (6)).

hydrolysis value), and only a slight increase in hexosemonophosphate. In experiments in which the muscles were kept in the iodoacetate solution until rigor developed, there occurred a marked rise in hexosemonophosphate and an accumulation of hexosediphosphate.

Effect of Epinephrine on Muscles Poisoned with Iodoacetate—In the experiments in Table II two groups of matched muscles were

TABLE I

Effect of Iodoacetate on Phosphate Distribution in Anaerobic Frog Muscle

The time of exposure was 60 minutes. The values are given in mg. per gm. of muscle.

Experiment No.	Inorganic P	Phosphocreatine P	P after hydrolysis in N HCl			Total P	Hexosemonophosphate		
			0 min.	8 min.	30 min.		Hexose	P found	P calculated
Without iodoacetate									
1-a	31	53	84	112	113	131	20	6	3
2-a	24	66	90	119	121	137	22		4
3-a	37	62	99	127	130	146	29		5
4-a			88	113	116	137	19		3
	30	60	90	118 +28	120 +2	138 +18	22		4
With iodoacetate (2.7×10^{-4} M)									
1-b	36	47	83	113	115	135	32	10	6
2-b	27	64	91	119	121	138	20		3
3-b	34	52	87	116	121	150	53		9
4-b			88	112	113	142	22		4
	32	54	87	115 +28	117 +2	141 +24	31		6

kept anaerobically in iodoacetate 1:20,000, epinephrine being added to one group. The experiments lasted 70 to 90 minutes (in order to produce a sufficiently marked effect of epinephrine), while according to Table I 60 minutes would have been preferable in order to keep the changes produced by iodoacetate itself as small as possible. The muscles treated with epinephrine showed a decrease in inorganic phosphate and increase in hexosemono-

phosphate; *i.e.*, epinephrine had a similar effect on phosphate distribution in muscles poisoned with iodoacetate to that in unpoisoned muscles. On an average the inorganic P diminished 7 mg. per cent, while the P esterified as hexosemonophosphate increased 8 mg. per cent.

The only difference in the action of epinephrine on unpoisoned and on poisoned muscle was that it could not cause an increase in

TABLE II

Effect of Epinephrine on Anaerobic Frog Muscle Poisoned with Iodoacetate

The values are given in mg. per 100 gm. of muscle.

Experiment No.	Time of exposure	Inorganic P	Phosphocreatine P	P after hydrolysis in N HCl			Total P	Hexosemonophosphate		
				0 min.	8 min.	30 min.		Hexose	P found	P calculated
Iodoacetate (2.7×10^{-4} M)										
*	min.									
5-a	70	29	43	72	100	103	121	41	9	7
6-a	80	29	43	72	96	102	125	76	13	13
7-a	80	33	45	78	105	110	136	57	12	10
8-a	90	25	37	62	86	94	126	81	13	14
		29	42	71	97 +26	102 +5	127 +25	64	12	11
Iodoacetate + epinephrine (1.1×10^{-6} M)										
5-b	70	23	36	59	85	92	122	101	18	17
6-b	80	26	40	66	91	94	128	112	21	19
7-b	80	23	42	65	96	102	140	106	21	18
8-b	90	16	34	50	84	92	123	116		20
		22	38	60	89 +29	95 +6	128 +33	109		19

lactic acid in the latter. In two experiments the following values for lactic acid were obtained: muscles kept for $1\frac{1}{2}$ and 2 hours in epinephrine, 29 and 40 mg. per cent; muscles kept for $1\frac{1}{2}$ and 2 hours in epinephrine plus iodoacetate, 11 and 14 mg. per cent. These experiments show the suppression of lactic acid formation by iodoacetate and from what is known about its action one would expect fructosediphosphate and dihydroxyacetonephos-

phate (8) to accumulate instead of lactic acid. Furthermore, phosphocreatine should diminish to the extent to which these esters accumulate. In contrast to the Embden ester, these two

TABLE III

Effect of Dinitrophenol and Caffeine on Phosphate Distribution in Anaerobic Frog Muscle

The values are given in mg. per 100 gm. of muscle.

Experiment No.	Time of exposure	Concentration of drug	Inorganic P	Phosphocreatine P	P after hydrolysis in N HCl			Total P	Hexosemono-phosphate			Lactic acid
					0 min.	8 min.	30 min.		Hexose	P found	P calculated	
Without addition of drugs												
9-a	65		42	62	104	140	143	169	32	6	6	
10-a	65		34	58	92	128	132	151				31
11-a	65		27	59	86	121	123	143	28		5	44
12-a	65		41	57	98	132	134	159	21		4	41
13-a	120		44	48	92	128	131	151	27	6	5	45
14-a	120		30	60	90	117	119	135	20		4	(25)*
				36	57	93	127 +34	130 +3	151 +21	25		4
With addition of drugs (D. = dinitrophenol; C. = caffeine)												
9-b	65	D. 3.5×10^{-6}	64	40	104	140	145	170	47	9	8	
10-b	65	" 7.1×10^{-6}	59	37	96	129	132	154				54
11-b	65	" 3.5×10^{-6}	52	40	92	125	127	147	31		5	65
12-b	65	C. 1.2×10^{-3}	51	45	96	131	135	158	34		6	59
13-b	120	D. 7.1×10^{-6}	68	22	90	123	126	152	33	8	6	109
14-b	120	C. 1.2×10^{-3}	56	34	90	116	118	138	24		4	(50)*
			58	36	94	127 +33	130 +3	153 +23	33		6	72

* Ringer's solution, changed at start of anaerobic period.

keto-esters are easily hydrolyzed in acid. It may be seen in Table II that the difference between the 0 and 30 minute hydrolysis value was larger in the experiments with epinephrine than in those without. That these two esters were formed was also sug-

gested by determinations of the ketose content (by means of the Seliwanoff reaction as modified by Roe (9)) which gave slightly higher values in the muscles treated with epinephrine than in those without epinephrine. Finally, as shown in Table II, there was a decrease in phosphocreatine in the muscles treated with epinephrine amounting to 4 mg. per cent.

It has not been possible to increase hexosemonophosphate in

TABLE IV

Effect of Dinitrophenol on Anaerobic Frog Muscle Poisoned with Iodoacetate

The values are given in mg. per 100 gm. of muscle.

Experiment No.	Time of exposure	Inorganic P	Phosphocreatine P	P after hydrolysis in N HCl			Total P	Hexosemonophosphate		
				0 min.	8 min.	30 min.		Hexose	P found	P calculated
Iodoacetate (2.7×10^{-4} M)										
	min.									
14-a	55	36	44	80	110	113	135	27	8	5
15-a	57	31	47	78	103	105	128	38	8	7
16-a	72	38	44	82	109	115	140	43	8	7
17-a	75	33	42	75	102	105	126	38	7	7
		34	44	78	106 +28	109 +3	135 +26	38		6
Iodoacetate + dinitrophenol (3.5×10^{-4} M)										
14-b	55	38	36	74	90	101	136	51		9
15-b	57	35	22	57	93	102	135	47	12	8
16-b	72	41	30	71	89	104	141	56	11	10
17-b	75	40	22	62	103	106	127	38	7	7
		39	27	66	94 +28	103 +9	135 +32	48		8

unpoisoned muscles without producing at the same time some increase in lactic acid. The experiments in Table II must therefore be contrasted with experiments in which the opposite is accomplished; namely, a marked rise in lactic acid without much change in hexosemonophosphate.

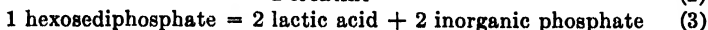
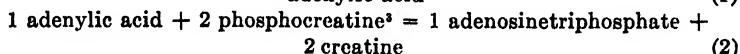
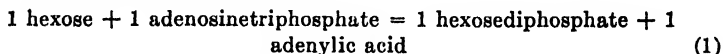
Effect of Dinitrophenol and Caffeine—Ehrenfest and Ronzoni (10) found that the optimum concentration of 2,4-dinitrophenol

for frog tissues at 25° and pH 7.5 was 2.7×10^{-5} M, a concentration which stimulated the rate of anaerobic lactic acid production of muscle very markedly. In the experiments in Table III, a concentration of dinitrophenol considerably below that required to give maximal effects was used, because rigor sets in very soon when this dye is added in large doses to muscles poisoned with iodoacetate.

During 1 to 2 hours of incubation in dinitrophenol or caffeine (in the low concentrations used) the hexosemonophosphate content of muscle showed only a slight change.² The lactate production was increased in each experiment and at the same time the phosphocreatine content of muscle showed a marked drop. In these muscles (which were not treated with iodoacetate, Table III), the decrease in phosphocreatine (−21 mg. per cent) was balanced by the increase in inorganic phosphate (+22 mg. per cent). In contrast to this finding, the breakdown of phosphocreatine in muscles poisoned with iodoacetate (Table IV) was not accompanied by the liberation of an equivalent amount of inorganic phosphate. Instead, an organic phosphate easily hydrolyzed in acid and giving a strong ketose test was found to accumulate, presumably a mixture of hexosediphosphate and dihydroxyacetonephosphate.

DISCUSSION

The present experiments can best be interpreted on the basis of the following reactions (1, 2).



² With higher concentrations of dinitrophenol and longer time of exposure the hexosemonophosphate content of muscle showed a rise.

³ Parnas (2) has shown that phosphopyruvic acid can replace phosphocreatine in reaction (2). This seems to occur after the initial period of lactic acid formation is over, as shown by the fact that phosphocreatine disappears much more rapidly in the beginning than at the end of a tetanic contraction. The conditions under which one or the other reaction occurs in non-contracting muscle have not been made clear. The purpose of both reactions has, however, been established; namely, to prevent the accumulation of free adenylic acid which would be immediately deaminized, resulting in a loss of coenzyme.

In muscle treated with dinitrophenol or caffeine, where the reactions can proceed to stage (3), one finds that as much inorganic phosphate is liberated as phosphocreatine disappears. That phosphocreatine actually enters into reaction (2) (the so called Lohmann reaction) and is not merely broken down to creatine and inorganic phosphate is shown by the outcome of the experiments with iodoacetate-poisoned muscle. Iodoacetic acid by blocking reaction (3), prevents the liberation of inorganic phosphate from hexosediphosphate, while reaction (2) proceeds as before, so that much more phosphocreatine disappears than inorganic phosphate appears (see Table IV). Caffeine and dinitrophenol apparently accelerate reaction (1), and thereby increase the rate of lactic acid formation.

The accumulation of hexosemonophosphate (in muscle treated with epinephrine and iodoacetate, see Table II) is accompanied by a decrease in inorganic phosphate without much change in phosphocreatine. The assumption is that adenosinetriphosphate does not furnish its phosphate for the formation of the monoester, because a reaction similar to that in (1) would be followed by reaction (2), resulting in a decrease in phosphocreatine without any change in inorganic phosphate. The experimental findings point to the formation of hexosemonophosphate from inorganic phosphate through an as yet unexplained mechanism.⁴

SUMMARY

1. The accumulation of hexosemonophosphate in anaerobic frog muscle, treated with epinephrine and iodoacetate, is accompanied by an equivalent decrease in inorganic phosphate and only a slight drop in phosphocreatine.

2. The increased lactic acid formation in anaerobic frog muscle—under the influence of small doses of dinitrophenol or caffeine and short times of exposure—results in a marked decrease in phosphocreatine and an equivalent increase in inorganic phosphate without the accumulation of significant amounts of hexosemonophosphate.

3. Under the same conditions as in (2), but with iodoacetate

⁴ Since the above was written, it has been shown (11) that adenylic acid acts as a carrier for the inorganic phosphate in the formation of hexosemonophosphate.

poisoning, hexosediphosphate accumulates instead of lactic acid and the decrease in phosphocreatine is not accompanied by the liberation of inorganic phosphate; *i.e.*, the phosphate split off from phosphocreatine is locked away mainly as hexosediphosphate.

4. It is concluded that hexosediphosphate is formed by phosphorylation of hexose with the labile phosphoric acid groups of adenosinetriphosphate, followed by a transfer of phosphocreatine phosphate to adenylic acid. Hexosemonophosphate is formed at the expense of inorganic phosphate.

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AN UNUSUAL CASE OF ESTERIFICATION IN MUSCLE*

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It has been shown in the preceding paper (1) that dinitrophenol and caffeine in small doses accelerate lactic acid formation in anaerobic frog muscle without increasing the hexosemonophosphate content of muscle. Epinephrine, on the other hand, has much more effect on hexosemonophosphate than on lactic acid. These results suggested a means of finding out whether or not there was any connection between hexosemonophosphate and lactic acid formation. If the two processes were independent of each other, one would expect to get the sum of the separate effects of each drug. This was the case when epinephrine and caffeine were combined. With dinitrophenol an unexpected result was obtained, since it intensified very markedly the esterifying action of epinephrine.

EXPERIMENTAL

The methods used were the same as those reported in the preceding paper. The results are shown in Table I. Epinephrine alone had its usual effect on phosphate distribution and produced an average level of 106 mg. per cent of hexosemonophosphate in 2 hours. Epinephrine and dinitrophenol acting together for 2 hours produced in most cases a level of hexosemonophosphate of 200 mg. per cent or more, while dinitrophenol acting alone for this length of time had hardly any effect on hexosemonophosphate (see Table III of the preceding paper). Dinitrophenol, therefore, greatly intensifies the esterifying action of epinephrine. During 3 hours of exposure to both drugs hexosemonophosphate levels

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TABLE I

Effect of Epinephrine Plus Dinitrophenol or Caffeine on Anaerobic Frog Muscle

The time of exposure was 120 minutes. The values are given in mg. per 100 gm. of muscle.

Experiment No.	Inorganic P	Phosphocreatine P	P after hydrolysis in N HCl		Total P	Hexosemonophosphate			Lactic acid
			0 min.	8 min.		Hexose	P found	P calculated	
Epinephrine alone (1.1×10^{-5} M)									
1-a			73	95	128	117	20	20	33
2-a			71	95	132	110	17	19	26
3-a	26	52	78	112	152	129	23	22	61
4-a	28	60	88	118	147	74		13	31
5-a	31	53	84	118	157	110		19	59
6-a	31	61	92	115	152	96	17	16	46
7-a			77	195	143	109	17	17	
	29	56	80	108 +28	144 +36	106		18	43
Epinephrine + dinitrophenol (7.1×10^{-6} M)									
1-b			56	78	131	234	38	40	81
2-b			57	80	137	236	33	40	113
3-b	37	28	65	97	154	241	40	41	183
4-b	39	31	70	109	148	142		25	62
			62	91 +29	142 +51	213		37	110
Epinephrine + caffeine (1.2×10^{-3} M)									
5-b	42	45	87	122	161	108		19	72
6-b	51	32	83	110	153	136	23	23	134
7-b			76	105	148	102	15	16	
			82	112 +30	154 +42	115		19	

of 288 and 267 mg. per cent were reached, corresponding to 50 and 46 mg. per cent of organic P or to one-third of the total acid-soluble P. These are by far the highest hexosemonophosphate values observed so far in muscle.

Irrespective of this large accumulation of hexosemonophosphate, dinitrophenol had its usual accelerating effect on lactic acid formation. Caffeine also accelerated lactic acid formation, but, in contrast to dinitrophenol, it did not enhance hexosemonophosphate formation in the presence of epinephrine. The two processes can apparently vary, within rather wide limits, independently of each other and there appears to be no connection, in intact muscle at least, between hexosemonophosphate and lactic acid formation.

It was shown in the preceding paper that dinitrophenol causes

TABLE II

Aerobic Disappearance of Hexosemonophosphate Accumulated under Action of Epinephrine Plus Dinitrophenol

All the values are given in mg. per 100 gm. of muscle.

After anaerobic period (2 hrs.)		After subsequent aerobic period (2 hrs.)		Carbohydrate equivalent of O ₂ consumption
Hexosemonophosphate as hexose	Lactic acid	Hexosemonophosphate as hexose	Lactic acid	
215		64		41
241	86	137	109	46
218	109	102	105	
236		108		
234	66	84	92	35
224	87	76	111	50
228	49	114	64	
228	80	98	96	43

a decrease in phosphocreatine and an increase in inorganic phosphate. The same effects are seen in the presence of epinephrine, but they are apparently not responsible for the enhancement of epinephrine action, because caffeine has the same effect on phosphocreatine but does not intensify hexosemonophosphate formation.

No explanation has been found for the combined action of dinitrophenol and epinephrine. That the accumulation of hexosemonophosphate is of a reversible nature is shown in the experiments in Table II. Of two groups of matched muscles, treated with dinitrophenol (7.1×10^{-6} M) and epinephrine (1.1×10^{-6}

m), one was analyzed after 2 hours of anaerobiosis, while the other was transferred to fresh Ringer's solution without drugs and allowed to recover for 2 hours in oxygen. Hexosemonophosphate disappeared very rapidly and this was accompanied by the liberation of inorganic phosphate. Lactic acid increased slightly during the aerobic period, presumably because the muscles which were stimulated to a high rate of O_2 consumption by dinitrophenol had too large a diameter for complete diffusion of oxygen. In some experiments the O_2 consumption was measured in a Warburg apparatus and it was found that 3 times as much hexosemonophosphate disappeared as could be accounted for by the total O_2 consumption. A similar observation was made on a previous occasion (2), when part of the disappearing hexosemonophosphate was accounted for as glycogen.

SUMMARY

1. Dinitrophenol, which in the concentrations used did not significantly alter the hexosemonophosphate content of anaerobic frog muscle, greatly intensified the esterifying action of epinephrine, so that twice as much hexosemonophosphate was formed by the combined treatment with dinitrophenol and epinephrine as with epinephrine alone. In some cases the hexosemonophosphate fraction contained one-third of the acid-soluble P, a 10-fold increase over the normal. Irrespective of this large esterification, dinitrophenol produced its usual accelerating effect on lactic acid formation and caused its usual decrease in phosphocreatine.

2. The combined effect of caffeine and epinephrine corresponded to the sum of the separate action of these drugs; *i.e.*, caffeine did not enhance the esterifying action of epinephrine.

3. Hexosemonophosphate, accumulated under the action of dinitrophenol and epinephrine, disappeared rapidly when oxygen was admitted, and the total oxygen consumption of muscle accounted for only one-third of the disappearing ester.

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THE METABOLISM OF SODIUM ACETOACETATE INTRAVENOUSLY INJECTED INTO DOGS*

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Of the fundamental questions concerning the phenomenon of ketosis, one, the origin of the "acetone bodies" from fats, is generally conceded, although the details *in vivo* are not clearly established. The other question, why their oxidation stops at this stage, appears to be answered by the relation of ketosis to deficient carbohydrate combustion first noted by Hirschfeld (1) in 1895. But the nature or mechanism of this relation remains perplexing and unsolved. Some investigators (2) take the view that carbohydrate prevents or abolishes ketosis merely by its replacement of fat in the materials oxidized. Others have taken the view that a closer chemical relationship exists, and that "fats burn in the fire of carbohydrates" (3). Discovery of the fact that reducing sugars, when oxidized by H_2O_2 (4) or O_2 (5) *in vitro*, cause the oxidation of acetoacetate, led Shaffer to infer the possibility that a similar reaction may explain the antiketogenic action of carbohydrate in human metabolism (6). His analysis showed in most cases a fair agreement with his hypothesis as to a quantitative relation between ketogenic and antiketogenic factors. But apart from the rather striking correspondence between theory and calculation of the quantitative factors in human metabolism (a correspondence which appears to hold also for the fasting ketosis of some other Primates (7)), and apart from the possible *in vitro* analogy mentioned above, there is no very convincing evidence to decide the

* A preliminary report of the experiments described in this paper was presented in December, 1925, before the American Society of Biological Chemists (*Proc. Am. Soc. Biol. Chem.*, 7, 44 (1925); *J. Biol. Chem.*, 67 (1926)).

nature of antiketogenesis. It is known that other animals do not develop a ketosis to a degree comparable to that seen in man and that some human subjects appear to be exceptional (8). Although insulin relief of ketosis in diabetes is approximately parallel with its restoration of carbohydrate combustion, attempts to demonstrate an effect of insulin upon either the rate of acetone production by the perfused liver (9) or upon the rate of acetoacetate combustion by decapitated cats (10) have yielded negative results.

It should be noted that our present knowledge concerning the metabolism of fats has been gained chiefly through the study of formation and further metabolism of the acetone bodies. Thus, only fatty acids of an even number of carbon atoms yield acetone bodies, both when perfused through isolated organs (11) and when given to the intact animal (12, 13). The same result is obtained by oxidation *in vitro* (14). This and the observation that both acetoacetic and β -hydroxybutyric acids are always formed (both of which represent oxidation at the β -carbon atom) have led to the view that oxidation of fatty acids can and does occur at the β -carbon atom. Whether the keto or the hydroxy acid is the primary metabolic product from the fatty acid, or whether oxidation is preceded by desaturation, is of considerable theoretical importance. Crotonic acid yields acetoacetic and β -hydroxybutyric acids (13, 15). Acetoacetic acid is readily converted into β -hydroxybutyric acid, although the latter does not appear to be so readily converted into acetoacetic acid (12, 13). Attempts to answer the question¹ have been made (a) by studying the rate of metabolism of the two acetone acids (Neubauer, Blum, Dakin, Marriott, Wilder), (b) by noting the rate and extent of formation of the two acids in diabetic human subjects (Neubauer, Kennaway), and (c) by noting the relative rate of appearance of the two in the blood following the administration of a fatty acid to normal animals (Marriott). The relation of fat to the carbohydrate metabolism has been studied almost entirely by a determination of the acetone bodies. Even in the more recent work with pituitary and other organ extracts, it has been assumed that the appearance of acetone bodies is an indication of the effect of these extracts on the fat or carbohydrate metabolism.

¹ For a complete review of this subject see Dakin (16).

Since the metabolism of acetone bodies has assumed such an important rôle in the development of theories concerning fat metabolism, it is essential to have accurate quantitative data concerning their rate of metabolism and the extent of interconversion of acetoacetic and β -hydroxybutyric acids. Many of the older data are not sufficiently accurate, either because of the analytical methods employed or because they do not represent *equilibrium* conditions. Equilibrium can be attained either by the continued intravenous injection at a constant rate, as used by Wilder (17), or by a single intravenous injection of the nephrectomized animal, as used by Chaikoff and Soskin (18). We prefer the method of Wilder, since it involves fewer operative procedures.

The experiments here reported were undertaken (in 1924) in order to learn whether the rate of disposal of acetoacetic acid and the extent of its conversion into β -hydroxybutyric acid, when injected intravenously into dogs, are modified by the state of the carbohydrate metabolism. It was realized that dogs may not be very suitable for the purpose because of their known resistance to the development of ketosis. This difficulty, it was thought, might be overcome by greatly increasing the rate of acetoacetate injection. We injected it continuously for 6 to 18 hours. The results demonstrate, in confirmation of a large mass of earlier work,² a remarkable ability of the dog to dispose of acetoacetate, perhaps mostly by direct oxidation. This tolerance is only to a slight extent, if at all, influenced by insulin or phlorhizin injection under the conditions used. The rate of disappearance of acetoacetate rises with the rate of injection, only a fraction appearing in the urine, part as the keto and part as the hydroxy acid. The ratio of acetoacetic acid to β -hydroxybutyric acid appearing in the urine is a function of the rate of excretion.

Our conclusions with respect to the effect of insulin have since been confirmed by Rosenthal (20) and Chaikoff and Soskin. The experimental conditions, however, were quite different.

EXPERIMENTAL

Apparatus—The apparatus was similar in principle to that of Burn and Dale (10). It consisted of a large reservoir (of 500

² The earlier work is reviewed by Allen and Wishart (19).

or 1000 cc. capacity, calibrated at 10 cc. intervals) containing the acetoacetate solution, an oil reservoir, and a device for regulating the pressure of air above the surface of the oil. Oil was forced from the reservoir through capillary tubes into the top of the acetoacetate burette. The solution thus displaced by the oil was conducted through flexible pure gum tubing, through a glass T-tube, and finally through a silver cannula into the vein.

Sodium Acetoacetate—Sodium acetoacetate was prepared by saponification of the redistilled ethyl ester with an excess of alkali, acidification, extraction with ether, and finally preparation of the sodium salt. The solution was filtered, phenol red was added, and the reaction was adjusted to about pH 7.5. The solution was immediately placed in the refrigerator. It was analyzed and diluted to the strength desired.

Procedure

The animals chosen for these experiments were healthy, well fed, adult dogs. With one exception, they were males. Practically all were fat; a few were very fat. Food was withheld during the 24 hours preceding the experiment.

The animal was catheterized and 60 mg. of anytal per kilo of body weight were injected intraperitoneally. The catheter was allowed to stay in place throughout the experiment. The silver cannula was next inserted either in the femoral or saphenous vein. Injection of the solution was then started. The rate of flow was regulated as follows: The injection at the beginning was made with a high air pressure in the oil reservoir. The rate of injection and the manometer readings were recorded. The air pressure was then reduced and the rate of injection and the pressure were again recorded. From these data, obtained over a period of about 20 minutes, it was possible to calculate approximately the pressure necessary to bring about the desired flow of solution. Burette and manometer readings were made at about 10 minute intervals and, if necessary, adjustments of the air pressure were made to bring the flow to the desired rate.

The injections in the first five experiments were accompanied by a very severe alkalosis (see Tables II and III, Fig. 4). An attempt was made to prevent this occurrence in subsequent experiments by simultaneously injecting at a constant rate sufficient

HCl solution to neutralize about two-thirds of the base of the acetoacetate (see Tables IV and V). The acid was introduced through the T-tube and was allowed to mix with the acetoacetate in the tubing leading to the cannula. Although the alkalinity of the urine decreased (see Table V), no apparent improvement in the condition of the animals so treated was noted.

Body temperature was maintained by means of a thermostatically controlled electric hot pad. In a few instances the temperature rose instead of falling. Abnormally high temperatures were reduced by applying ice packs.

Analytical Methods

Analyses were carried out on urine, blood, tissues, and exhaled air.

Urine was collected in a graduated cylinder during the half hour or hour periods and, after being transferred to a bottle, was immediately placed in the refrigerator.

Acetoacetic acid and acetone were determined by distilling an aliquot suitably diluted and acidified. The distillate was redistilled from sodium peroxide. Acetone was determined by the Messinger method. β -Hydroxybutyric acid was determined on the copper-calcium (Van Slyke) filtrate of the urine by the method of Shaffer and Marriott (21).

The base, bound by the excreted carbonates, was determined as follows: 10 cc. of urine, 5 cc. of a saturated solution of neutral potassium oxalate, and methyl orange and phenolphthalein indicators were placed in an Erlenmeyer flask. An excess of standard HCl was added, and air was blown through the solution to remove all CO_2 . Standard NaOH was then added to the phenolphthalein end-point. HCl under these conditions releases carbonic acid and thus permits an approximate measure of the base bound by the carbonates. Since the urine was distinctly alkaline (pH 7.5 to 8.5), carbonic acid was present largely as bicarbonate. The titration, therefore, was a fairly accurate method of determining the carbonate content.

The total CO_2 of urine was determined by aerating an acidified aliquot, as described by Friedemann and Kendall (22).

Acetoacetic acid was determined in the tissues of four animals within 15 minutes after the injection of acetoacetate was dis-

continued. The dogs were rapidly bled from the carotid artery, the blood being collected in a beaker containing potassium oxalate. The tissues were hashed in a cold meat grinder; 50 to 100 gm. samples were weighed out immediately and well mixed with 100 cc. of ice-cold $N H_2SO_4$. This mixture was then transferred to an 800 cc. Kjeldahl flask. The distillate was redistilled from Na_2O_2 . Acetone was determined as described above.

Acetone was determined in the exhaled air of only two animals,

TABLE I
Summary of Experiments

Dog No.	Weight	Experimental conditions	Aceto-acetate solution	Maximum and minimum rate of injection
	<i>kg.</i>		<i>M</i>	<i>mm per kg. per hr.</i>
1	19.2	Normal (5, 6); insulin (7-11)	0.91	3.97-4.35
2	5.6	“ glucose (2, 3); insulin (4-10)	0.90	3.95-5.64
4	12.5	“ (4-15)	0.50	3.26-4.68
5	19.6	“ (5, 6); phlorhizin (7-14)	0.51	2.86-3.83
6	8.0	Depancreatized; insulin, food and glucose (4-9)	0.47	2.13-2.68
7	12.8	Normal (2-5)	0.59	7.10-7.60
8	8.2	“ (4-10); glucose (11-13); insulin and glucose (14, 15)	0.25	2.07-2.38
9	9.2	Fasting and phlorhizin (4-7); insulin and glucose (8-10)	0.30	2.28-2.61
10	15.6	Fasting (5); phlorhizin (6-13)	0.52	2.27-2.60
11	12.0	“ depancreatized (2, 3); glucose and insulin (4, 5)	0.26	3.25-3.41
12	18.5	Fasting and phlorhizin (4, 5); like Dog 9	0.25	2.78-2.90

The figures in parentheses indicate the urine collection periods. For details see Tables II to V.

Dogs 7 and 10. A cannula was introduced into the trachea, and the expired air was passed through two large gas-washing bottles. Each of the bottles contained 300 cc. of dilute alkaline hypoiodite solution. Only one such bottle was found necessary for complete absorption.

Results

The conditions under which these experiments were conducted are summarized in Table I.

Unfortunately the onset of amytal narcosis was always accompanied by anuria. The diuretic effect of the injected salts, however, caused the flow of urine to begin 10 to 30 minutes after injection was started. In every instance the excreted urine contained both acetoacetic acid and β -hydroxybutyric acid. The total acetone bodies excreted gradually increased and finally reached the maximum after 2 to 3 hours.

The "Tolerance"

Results obtained after this equilibrium had been attained are shown in Fig. 1. The figure contains the experimental data of all

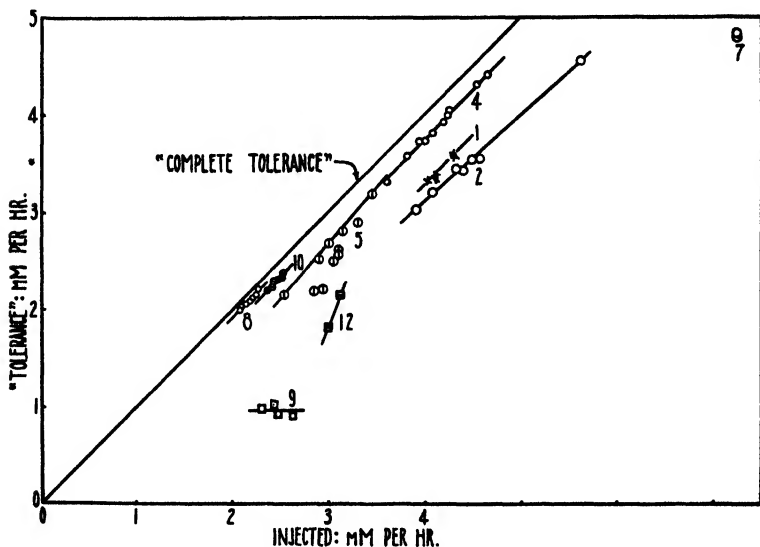


FIG. 1. The relation between the rate of injection and the rate of metabolism of acetoacetate. The figures on the curves refer to the dog numbers.

of the animals except two, Dogs 6 and 11. They are not included because the rate of injection and the quantity excreted are practically identical with those of Dogs 8 and 10. The injection rate per kilo per hour is plotted against the "tolerance" or the amount per kilo which is not excreted either as acetoacetate or β -hydroxybutyric acid during the hour, and which is assumed to be burned. If all of the injected material were retained, and none excreted, all

of the data would fall on the "complete tolerance" curve. As can be seen, however, some was always excreted, even at the injection rate of about 2 mm per kilo per hour.

The rates of injection during the course of each experiment varied rather widely (see Table I). The maximum variation in the case of Dog 4 (Table III), for example, was from 3.26 to 4.68 mm per kilo per hour. Furthermore, the rates varied irregularly from

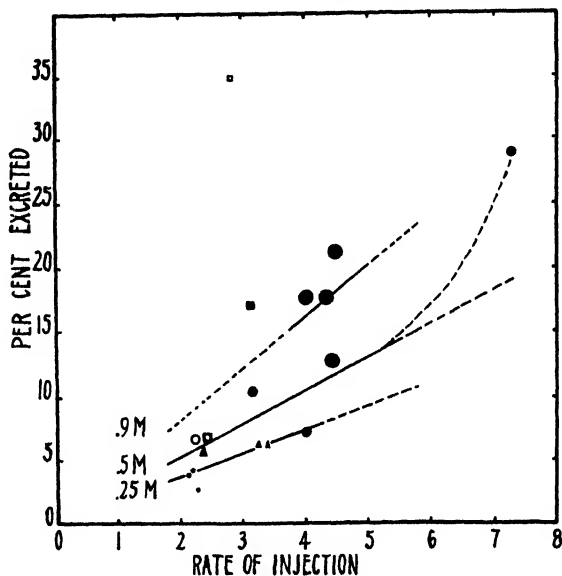


FIG. 2. The effect of concentration of injected sodium acetoacetate solution on the excretion of total acetone bodies. The points represent the average excretion during the periods indicated in Table I. The open and solid symbols indicate fasting and non-fasting animals, respectively. Normal animals are indicated by circles, depancreatized animals by triangles, and phlorhizinized animals by squares.

hour to hour. This fact is illustrated by the data from the same dog in which the rates varied as follows from the fifth to the sixteenth urine collection periods: 4.03, 3.82, 3.26, 4.68, 4.21, 4.26, 3.96, 4.55, 4.09, 3.84, 4.26 mm. In spite of these fluctuations the amount retained in the body is approximately proportional to the rate of injection per kilo of body weight. Indeed, practically all of the plotted data for any one of the normal animals fall on a straight line.

Effect of Concentration of Acetoacetate on Tolerance—Reference to Table I shows that the concentration of the acetoacetate solution injected into Dogs 1 and 2 was higher than that used in Dog 4. The tolerance was lower with the animals injected with the solu-

TABLE II

Injection of Neutral Acetoacetate and Glucose into Normal Dog; Effect of Insulin

Dog 2, male; 5.6 kilos. Small, poorly nourished. Amytal anesthesia. Injection of 0.904 M of freshly aerated, neutral acetoacetate and 20 per cent glucose begun at 10.30 a.m. Injections continued until 10.20 p.m., when the animal was given 125 cc. of 20 per cent glucose and 150 cc. of water by stomach tube and placed in a metabolism cage. Recovered and quite active the next morning. Acetoacetate strongly positive in urine 12 hours after termination of the injection, but negative after 24 hours.

* Urine collection period	Urine collected	0.904 M acetoacetate injected	Glucose injected	Acetone bodies in urine			Remarks
				Acetone-acetoacetic acid	β -Hydroxybutyric acid	Total acetone bodies	
	cc.	mM per hr.	mM per hr.	mM per hr.	mM per hr.	mM per hr.	
12 m.-1 p.m.	42.5	24.9	10.5	0.80	1.14	1.94	10.30 a.m. injection of acetoacetate begun
1-2 p.m.	68.5	25.0	10.2	1.28	1.85	3.13	10.32 a.m. injection of 20% glucose begun
2-3 "	65.0	24.9	10.9	1.29	1.97	3.26	
3-4 "	80.0	31.6	13.1	2.61	3.29	5.90	3.30 p.m., insulin 40 units
4-5 "	81.0	25.3	9.8	2.10	3.25	5.35	
5-6 "	110.0	25.8	9.8	2.51	3.25	5.76	
6-7 "	95.0	22.1	8.9	2.41	2.60	5.01	
7-8 "	93.0	23.0	9.2	2.32	2.69	5.01	
8-9 "	91.0	24.8	9.1	2.71	2.77	5.48	
9-10 "	82.0	24.4	9.1	2.42	2.57	4.99	

tion of higher concentration (see Fig. 1). The same relation between strength of solution and the tolerance is also noted in the case of Dogs 8 and 10. Dog 10 was injected with 0.5 M acetoacetate, while Dog 8 was injected with 0.25 M solution. The tolerance in the case of Dog 10 was lower than in the case of Dog 8.

The effect of concentration of solution is more strikingly shown in Fig. 2, in which the per cent of acetone bodies excreted is plotted against the rate of injection per kilo of body weight. It will be noted first of all that *from 3 to approximately 25 per cent of the injected material is excreted, depending upon the rate of injection and the concentration of the solution.* Although the results are quite irregular, the general trend of the curves indicates that the percentage excreted increases as the rate of injection increases. At a given injection rate the per cent excreted is higher or lower, depending upon the concentration of the solution. The cause of this increase cannot be directly determined from the results. It is not, apparently, a sweeping out effect, since the diuresis is not markedly greater in the dogs injected with solutions of higher concentration than in similar experiments in which animals were injected with a solution of lower concentration.

Another point to be noted from Figs. 1 and 2 is that the limit of tolerance is approximately 5 mm per kilo per hour; beyond this rate, as illustrated by Dog 7 (Table IV), the rate of excretion is unusually high.

Fasting does not apparently decrease the tolerance in normal or depancreatized dogs. Our results thus agree with those of Rosenthal and Chaikoff and Soskin. Fasting, however, greatly increases the effect of phlorhizin, as is shown in the results from Dogs 9 and 12 (see Figs. 1 and 2).

Effect of Pancreatectomy—Total pancreatectomy did not appear to decrease the tolerance. Our results are thus in accord with those of Burn and Dale and Chaikoff and Soskin.

Phlorhizin—A slight increase in excretion of total acetone bodies was noted immediately following the administration of phlorhizin to normal dogs. This was of relatively short duration and returned to normal as the sugar excretion diminished. We have already discussed the effect of fasting and phlorhizin on the tolerance.

Effect of Insulin—No effect of insulin was shown on normal animals by Burn and Dale and Robertson (23). From our data it is apparent that the tolerance is very high in normal animals. It is perhaps at its maximum and for that reason an increase in tolerance is not to be expected from insulin.

It may, however, have marked effect in animals in which the

tolerance is already low. This is illustrated by Dog 9, the results for which are shown in Fig. 3. Food was withheld from this animal and phlorhizin was given subcutaneously in oil for 3 days

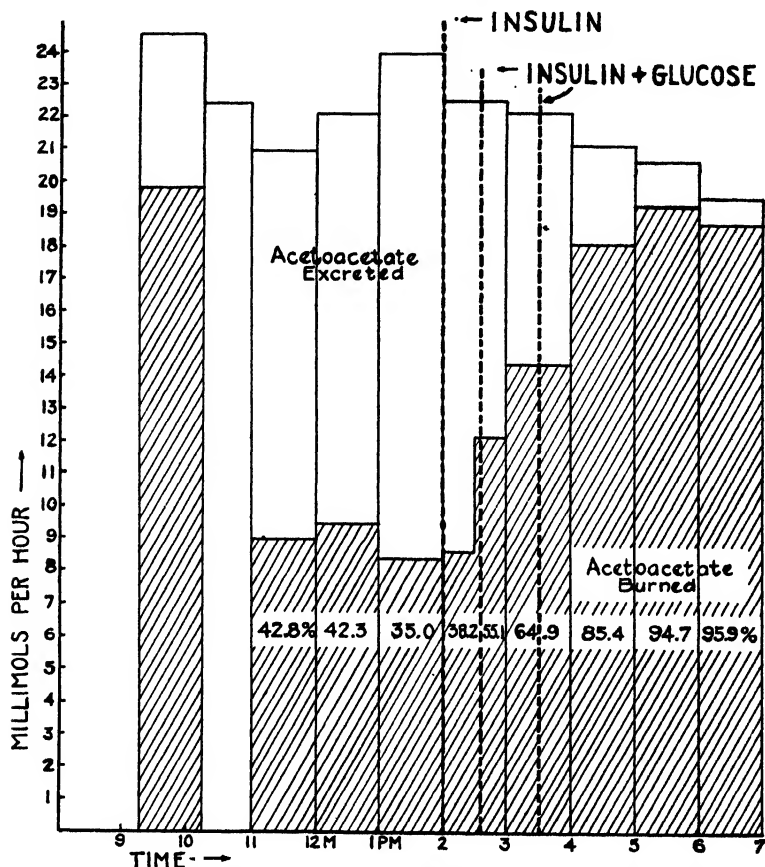


FIG. 3. Dog 9. Effect of insulin and insulin plus glucose on the metabolism of acetoacetate in a fasted phlorhizinized dog. 10 units of insulin were given in each of the three injections. 15 gm. of glucose in 50 per cent solution were injected intravenously at 2.37, 3.35, 4.20, and at 6.20 p.m.

preceding the experiment. 0.3 M acetoacetate solution was injected at an average rate of 2.5 mm per kilo per hour. At this rate about 5 per cent (see Fig. 2) was expected to be excreted, but from 35 to 53 per cent of the injected acetoacetate was excreted. In-

Injection of Neutral Acetoacetate into Normal Dog

Dog 4, female; 12.5 kilos. Healthy, fat, active. Fed at 10 a.m. on the previous day. Amytal by stomach tube. 0.504 m acetoacetate injection started at 11.26 a.m. The temperature fluctuated considerably during the latter part of the experiment; at the same time the animal appeared to be quite dehydrated. 300 cc. of water given by stomach tube at 7 p.m.; practically all vomited soon afterward. At 8.10 p.m. 370 cc. of saline given subcutaneously. At 9.10 p.m. ice pack applied. At 9.30 500 cc. of saline given subcutaneously. At 10.00 ice pack removed and heat applied. Ice pack applied again at 12.50 a.m.; removed at 1.35 a.m. Animal began to fail rapidly at about 2 a.m. and died at about 2.35 a.m. The acetoacetate solution flowed for some time after death. Animal was placed in refrigerator. Autopsy next morning showed all organs apparently normal. Kidney, spleen, liver, and pancreas, examined histologically, showed no pathological changes. Blood from heart immediately after death was viscous and dark. Blood was drawn for analysis at 11.20 a.m., 11.46 a.m., 3.15 p.m., and 2.40 a.m.

[illegible]

sulin was then given intravenously, followed by enough glucose to protect the animal. This resulted in an almost immediate rise in

TABLE IV

Injection of Neutral Acetoacetate and HCl into Normal Dog

Dog 7, male; 12.8 kilos. Very fat, healthy, and active. Amytal anesthesia at 10.43 a.m. 0.585 M acetoacetate and 0.5 N HCl were injected into the femoral vein. An attempt was made to inject these solutions in such a way as to neutralize at least half of the base, but not enough HCl appeared to have been injected, for it was found that the urine was still very alkaline. Hemoglobin appeared soon after the injection began and increased in quantity as the injection was continued. The animal began to fail at about 5 p.m. and died at 6.15 p.m. The muscles contained 0.618 mm of acetoacetate and acetone per 100 gm. 1 hour after death. The blood was viscous and slightly hemolyzed. Autopsy showed no pathological changes in the organs, except that the stomach was greatly distended with gas.

Urine collection period	Urine collected	0.585 M acetoacetate injected	Total volume of solutions (acetoacetate + 0.5 N HCl) injected	Acetone bodies in urine			Remarks
				Acetone-acetoacetic acid	β -Hydroxybutyric acid	Total acetone bodies	
	cc. per hr.	mm per hr.	cc. per hr.	mm per hr.	mm per hr.	mm per hr.	
2.15-3.00 p.m.	203	108	289	4.9	2.6	7.5	2.15 p.m. began injection of acetoacetate and HCl
3.00-3 35 "	480	97	237	15.7	9.3	25.0	
3.35-4.00 "	528	92	238	19.3	11.2	30.5	Much Hb in urine
4.00-4.30 "	500	92	229	20.1	10.6	30.7	Increased amount of Hb
4.30-5.00 "	380	91	227	13.4	7.7	21.1	Urine opaque with Hb, but no blood
5.00-5.30 "	254	88	230	7.1	3.4	10.5	5.30 p.m. 1.5 mm acetone per hr. in expired air
5.30-6.15 "	140	88	230	3.5	1.9	5.4	5.56 p.m. 1.5 mm acetone per hr. in expired air

tolerance, and within 3 hours the tolerance was normal. Unfortunately we have no control experiments in which only glucose was given, so that we cannot say that the result is due to insulin

TABLE V

Injection of Neutral Acetoacetate and HCl into Fasted Dog; Effect of Phlorhizin

Dog 10, male; 15.6 kilos. Very fat. Apparently quite old, since most of the teeth were gone. Animal not fed 48 hours preceding the experiment. Amytal at 6.38 a.m. Tracheal cannula inserted at 6.40 a.m. At 7.00 a.m. the injection of 1 per cent NaCl begun and by 9.00 300 cc. injected. At 9.02 injection of 0.52 M acetoacetate and 0.4 N HCl begun. 0.1 gm. of phlorhizin in Na_2CO_3 intravenously at 12.06 p.m.; 1.0 gm. of phlorhizin in oil subcutaneously at 12.26 p.m. Hemoglobinuria began at 1 p.m. and continued until the end of the experiment. The oxygen consumption and CO_2 production, as well as acetone in the expired air, were determined at various times. The injections were discontinued at 10.00 p.m. and the animal was bled from the carotid artery. The tissues were immediately ground and analyzed for acetone bodies.

Urine collection period	Urine collected	Analysis of urine							Respiratory data		
		0.52 M acetoacetate injected	Acetone bodies			Glucose	Total nitrogen	pH	Total expired air (0°, 760 mm.)	O ₂ consumed	CO ₂
			Acetone + acetoacetic acid	β -Hydroxybutyric acid	Total acetone bodies						
	cc. per hr.	mm per hr.	mm per hr.	mm per hr.	mm per hr.	mg. per hr.	mg. per hr.		l. per hr.	l. per hr.	l. per hr.
From bladder	192*						5942*	5.8			
7.45- 9.00 a.m.	27						440		39.3	4.35	3.38
9.00-10.00 "	89	40.0					426	7.1			
10.00-11.00 "	90	41.1	1.06	0.85	1.91		265	7.1			
11.00 a.m.-12.00 m.	160	35.4	1.20	1.18	2.38		269	7.4	45.5	5.11	4.64
12.00 m. - 1.00 p.m.	246	38.5	1.32	1.03	2.35	1950	255	7.4			
1.00- 2.00 p.m.	208	38.0	1.24	0.82	2.06	2350	216	7.5			
2.00- 3.00 "	205	38.0	1.30	0.87	2.17	2070	204	7.1	70.5	5.54	5.46
3.00- 4.00 "	232	36.9	1.52	0.96	2.48	1990	208	7.4			
4.00- 5.00 "	232	36.9	1.59	0.92	2.51	1820	200		72.5	5.50	5.27
5.00- 6.00 "	241	38.0	1.75	1.24	2.99	1700	206	7.3			
6.00- 7.00 "	227	39.5	1.55	1.44	2.99	1440	208	7.4			
7.00- 8.00 "	236	40.5	1.97	1.85	3.82	1110	225				
8.00- 9.00 "	189	39.0	1.35	1.29	2.64	700	209	7.4			
9.00-10.00 "	168	39.5	1.17	1.06	2.23	580	200				

* Total.

alone, but *this experiment unquestionably shows that available metabolizable carbohydrate can have an effect in an animal whose tolerance appears to be low.*

Expired Air—According to Marriott, acetone is not found in the expired air following an injection of acetoacetate. Chaikoff and Soskin, however, find that traces, considerably less than 1 per cent, are excreted by this route. That it is relatively small also in the case of dogs which receive acetoacetic acid intravenously over a long period of time is shown by the data from two dogs. Dog 7 (Table IV) received 88 mm of acetoacetate intravenously and excreted 1.5 mm as acetone in expired air; Dog 10 (Table V) received 37.5 mm and excreted 0.22 mm.

By inspection of the data in Table IV it will be noted that acetone determinations were made at the time when the urine excretion was rapidly falling and the retention of acetoacetic acid was rapidly increasing. The first determination was made 45 minutes and the second only 20 minutes preceding death. The conditions were favorable for the accumulation and decomposition of acetoacetic acid into acetone and, consequently, also for the excretion of acetone through the lungs. Dog 7, furthermore, as has already been pointed out, was injected at a rate beyond the "normal" tolerance limit. The results from this animal, therefore, probably represent the extreme. It is doubtful whether the excretion was much beyond 1 per cent in the other animals, as is indicated by the results from Dog 10. These were obtained several hours before death while the animal was still, as far as could be judged, normal.

Tissues—No data are available in the literature showing the acetone body content of the tissues in an animal which has been injected at a constant rate, and which presumably is in equilibrium with the injected material. Unfortunately we cannot present any such data for the total acetone body content of tissues; however, we have results showing the acetone plus acetoacetic acid content.

In Table VI is shown the acetone plus acetoacetic acid content of various tissues immediately after death. The rate of injection was relatively low and about the same in all of these animals: an average rate of about 2.3 mm per kilo per hour. The tissues of Dog 9, which was fasted and phlorhizinized, still contained large quan-

tities of acetoacetic acid at the time of death, soon after the tolerance had been apparently restored to normal by insulin and glucose (see Fig. 3). Although it is hardly fair to come to any conclusions in the absence of data for β -hydroxybutyric acid, it is apparent, at least as far as the acetone and acetoacetic acid contents are concerned, that the concentration of the latter two is lowest in the liver. *From a consideration of these data, it is immediately apparent that storage of acetone bodies, or any similar substances which might give rise to acetone by the usual analytical procedures, does not occur in the tissues.*³

Blood—Dogs 4 and 5 also afford an interesting study of the changes which occur in the blood. The results for acetoacetic

TABLE VI

Acetone and Acetoacetate Content of Tissues Immediately after Death

The rates of injection per kilo per hour during the period preceding death in Dogs 6, 8, 9, and 10 were 2.1, 2.4, 2.1, and 2.5 mm per kilo, respectively.

Tissue	Dog 6, depancreatized; insulin + glucose	Dog 8, normal; insulin + glucose	Dog 9, 3 days fasting + phlorhizin; insulin + glucose	Dog 10, normal; phlorhizin
	mm per kg.	mm per kg.	mm per kg.	mm per kg.
Blood.....	0.41	0.59	2.16	
Muscle.....	0.62	0.13	1.26	1.14
Liver.....	0.35	0.09	0.88	1.02
Heart.....	0.47			
Fat.....				0.65

acid (by chance, perhaps) coincide almost exactly. During the first few hours of injection the blood contained about 2.2 mm of acetoacetate per liter. It then rose to about 5 mm per liter and probably stayed at this level until the animal began to fail.

The CO₂ content of the blood was determined only in the case of Dog 5. Since all of the animals excreted much bicarbonate and, in general, behaved alike in other respects, the blood CO₂ content undoubtedly also varied in the same direction. The CO₂ content in this animal (see Fig. 4) rose from a normal value of 46 to 96

³ Respiratory data from one dog (Table V) show an increase in oxygen consumption sufficient to account for the acetoacetate not recovered.

volumes per cent within 4 hours and was still at this level at death at the end of 18 hours of continuous injection. The reaction of the blood collected under oil immediately after death was very alkaline, pH 7.8.

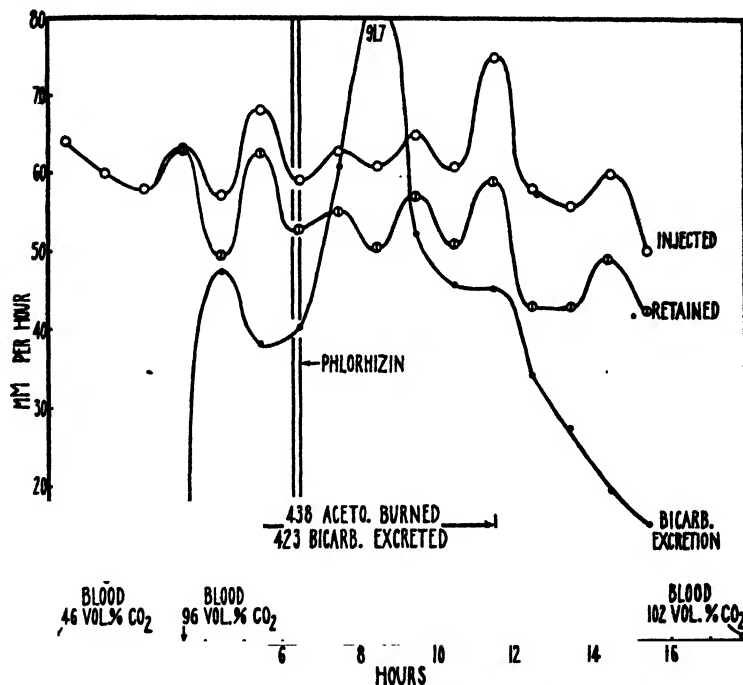


FIG. 4. Dog 5. The rate of injection and the quantity of acetoacetate retained, mm per kilo per hour. In this chart are shown also the rate of excretion of NaHCO_3 , the relation of acetoacetate retained to the NaHCO_3 excreted, and the changes in CO_2 content of the blood during the experiment. Injection of 0.509 M acetoacetate was begun at 12.00 m. Phlorhizin was given as follows: 1 gm. in oil subcutaneously at 6.20 p.m.; 0.1 gm. in Na_2CO_3 solution at 6.30 p.m.

The blood sugar remained fairly constant throughout these experiments.

Bicarbonate Content of Urine—Formation of bicarbonate from the metabolism of salts of organic acids has long been known and frequently observed qualitatively. Changes of the base and carbon dioxide content of urine and blood, following the injection of sodium lactate, were recently studied by Abramson and Eggle-

ton (24). These authors regard the formation of base from the lactate as proof of its metabolism.

The fact that the metabolism of sodium acetoacetate also leads to the formation of bicarbonate is illustrated by our data from Dogs 4 and 5 (Table III and Fig. 4). Total base and carbon dioxide were determined in the urine. Both of these dogs were normal and both were injected at a fairly rapid rate, the former at an average rate of about 4 mm and the latter at an average rate of about 3 mm per kilo per hour. Dog 5 received phlorhizin after the 6th hour. Following this injection, an exceptionally high diuresis was observed and, as was to be expected, large quantities of glucose were excreted. The urine was alkaline. Although the urine was immediately placed in stoppered bottles and kept in the refrigerator, no special precautions were taken to prevent loss of CO_2 while the specimens were being collected. It is not surprising, therefore, that a few were faintly alkaline to phenolphthalein. At this alkalinity CO_2 is present chiefly as bicarbonate. All of the urine specimens of Dog 4 were analyzed for total base and some for CO_2 . The amount of CO_2 was slightly lower than the base. On the whole, however, the agreement is fairly satisfactory. Because of this agreement only CO_2 was determined in the urine specimens from Dog 5.

The rate of bicarbonate excretion is shown by Fig. 4. Practically all of the acetoacetate was retained during the first 2 or 3 hours; only a relatively small amount of the base was excreted. During this period the CO_2 content of the blood increased, rising, as we have already stated, from an initial value of 46 to 96 volumes per cent in 3.5 hours, and from this time on remained at about the same level. The maximum CO_2 content of the blood coincided (a) with the point at which NaHCO_3 began to appear in large quantities in the urine and also (b) with the point at which the rate of excretion of total acetone bodies became approximately proportional to the rate of injection of acetoacetate. In other words, the animal appeared to reach equilibrium with respect to bicarbonate as well as to acetone bodies at about the same time.

After this equilibrium had been attained, the excretion of base, or NaHCO_3 , kept pace fairly well with the injection of acetoacetate (see the results from Dog 4, Table III). This is illustrated by the following calculations.

Dog 4—3.00 to 9.30 p.m. (Table III)

Total acetoacetate retained.....	323 mm
“ titrated base of urine.....	314 cc. N

Dog 5—4.00 p.m. to 12.00 a.m. (Fig. 4)

Total acetoacetate retained.....	438 mm
“ carbon dioxide in urine.....	423 “

A marked retention of the base was noted before the retention of acetoacetate and the failing condition of the animal became apparent.

Reduction of Acetoacetic Acid to β -Hydroxybutyric Acid

These two acids were always found together, even when the injection rate was low and the excretion of acetoacetic acid small. With such small quantities of β -hydroxybutyric acid as are excreted at the lower rates, it is exceedingly difficult to isolate either the calcium-zinc salt or to demonstrate levorotation. However, its presence was readily demonstrated by oxidation with acid-dichromate and slow redistillation with Na_2O_2 , according to the Shaffer-Marriott procedure.

The ratio of β -hydroxybutyric acid to acetoacetic acid, when the latter first appeared in the urine, was small, but it rapidly rose until it reached the maximum value for the given injection rate.

The results from the 81 urine collection periods indicated in Table I, representing approximate equilibrium conditions in the eleven dogs, are plotted in Fig. 5. The relative (Fig. 5, *A* and *C*) as well as the absolute (Fig. 5, *B* and *D*) amount of β -hydroxybutyric acid is represented. These quantities are compared with the rate of injection (Fig. 5, *A* and *B*) and also with the rate of excretion of acetone bodies (Fig. 5, *C* and *D*).

Considering the rate of injection (Fig. 5, *A*) first, it will be noted that the points for the ratio fall approximately within two curves. The relative amount of acetoacetate was relatively high when the injection rate was small, but it decreased, and β -hydroxybutyric acid increased, as the rate of injection was increased. The maximum was reached at an injection rate of about 3 mm per kilo per hour. At this point 2.1 times more of the hydroxy acid was excreted than of the keto acid. When acetoacetate was injected at this rate, the β -hydroxybutyric acid content of the urine became great enough to be demonstrable by optical rotation and by

other less sensitive means, such as isolation of calcium-zinc salts. It is interesting to note that Wilder (17) was able to demonstrate the first appearance of levorotation at an injection rate of 3.2 mm per kilo per hour.

When this rate was exceeded, the ratio no longer increased but

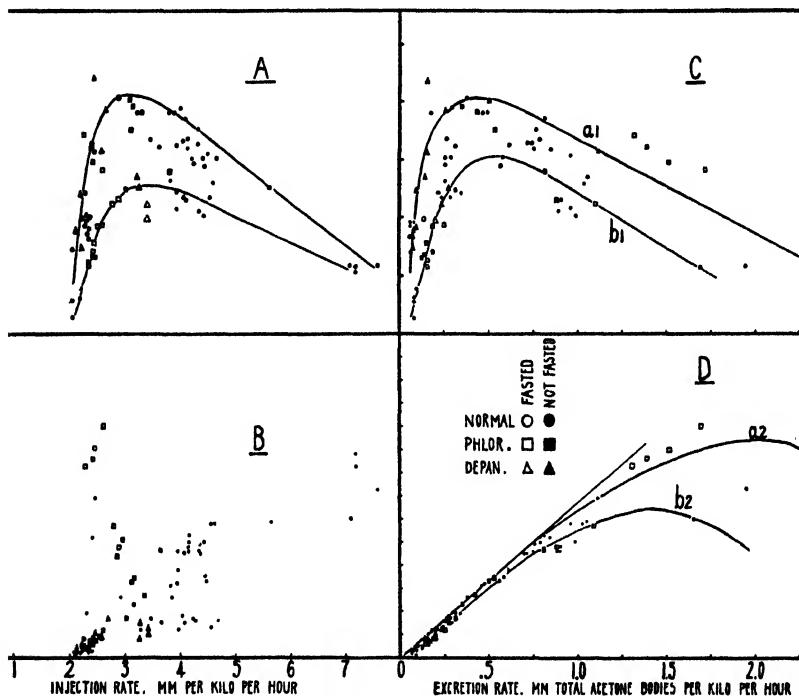


FIG. 5. Urinary acetone bodies. The ratio of β -hydroxybutyric acid to acetoacetic acid (A) and the rate of excretion of β -hydroxybutyric acid (B) compared with the injection rate. The ratio of β -hydroxybutyric acid to acetoacetic acid (C) and the rate of excretion of β -hydroxybutyric acid (D) compared with the rate of excretion.

decreased. It fell quite regularly and reached a value of about 0.5 at an injection rate of about 7.2 mm.

The excretion of β -hydroxybutyric acid when compared with the injection rate (Fig. 5, B) shows no striking correlation. Although there are many exceptions, a general trend toward an increase can be noted.

Greater uniformity is shown by Fig. 5, C and D, in which the

ratio and hydroxy acid excretion are correlated with the excretion rate. The extremes, as represented by Curves a1 and b1, are not so marked as in Fig. 5, A. The β -hydroxybutyric acid content of the urine is shown in Fig. 5, D. The β -hydroxybutyric acid output, as shown by Fig. 5, D, is remarkably regular.⁴

The extent of conversion into β -hydroxybutyric acid, as shown by Fig. 5, B and D, is not related to the total injected; *i.e.*, to the metabolized portion plus the excess which is excreted. If we assume that the excretion is proportional to the concentration in the blood and tissues, the results shown in Fig. 5, D indicate that the extent of conversion of the keto into the hydroxy acid is determined by the concentration of the unmetabolized portion.

The reduction to hydroxy acid appears not to be influenced by fasting, phlorhization, or pancreatectomy. Special attention is called to the four fasting, phlorhizin periods from Dog 9, open squares above Curve a1 (Fig. 5, C). Although these represent the minimum of "tolerance" (see Fig. 1), the extent of reduction of the excess, or unmetabolized portion, of the injected acetoacetate is still almost proportional to the rate of excretion of total acetone bodies. The rate of excretion in this experiment was relatively high because of impairment of the tolerance by fasting plus phlorhizin; the rate of injection was relatively slow. The four solid circles at the extreme right (Fig. 5, C) represent data from Dog 7, which received 7.2 mm per kilo per hour. Even though the quantity injected was excessively high and even though the resulting condition was decidedly more abnormal than in the other animals, the extent of reduction is fairly great. Under more normal conditions, with the same quantity of unmetabolized acetoacetate, the extent of reduction might perhaps have been greater than is indicated in Fig. 5, D.

Determinations by Chaikoff and Soskin indicate from 1 to 2 times as much β -hydroxybutyric acid as acetoacetic acid in the blood at equilibrium. Their data, expressed in terms of mm per liter, are shown in Fig. 6. Fig. 6 includes data from animals as follows: normal, normal fasted, normal eviscerated, diabetic fasted, and diabetic animals fasted and eviscerated. Insulin was given to some. The kidneys were removed in all before injec-

⁴ The significance of this will be discussed in a later paper.

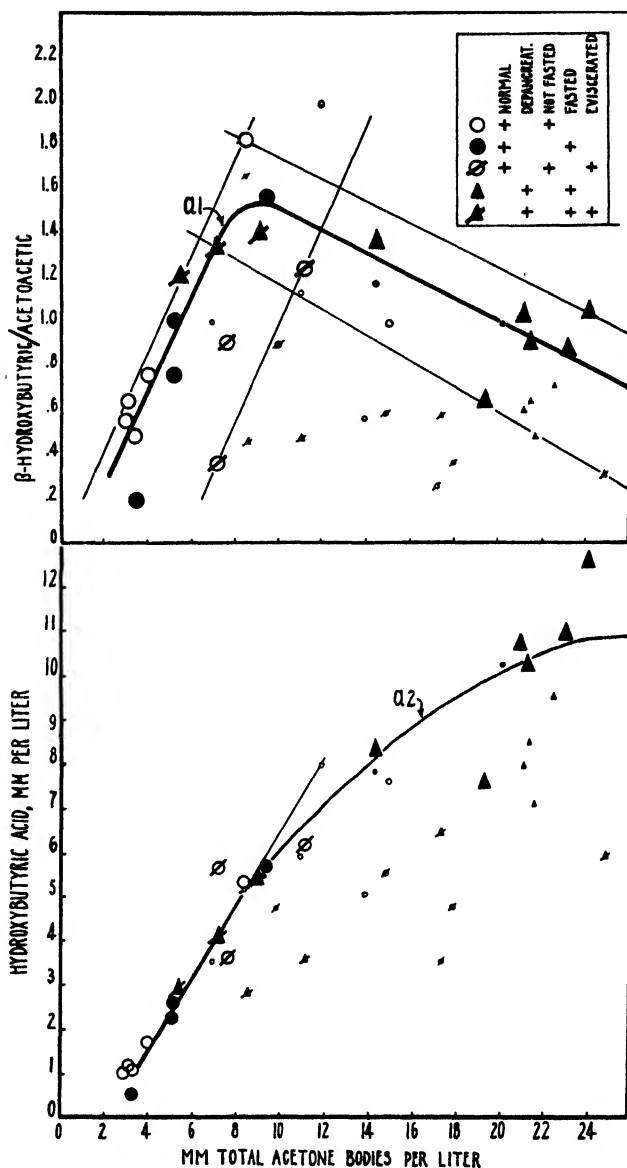


FIG. 6. Acetone bodies of the blood. Data of Chaikoff and Soskin. The small points represent analytical results obtained from 40 minutes up to, but not including, 2 hours after injection of 1 gm. per kilo of sodium acetoacetate. The large points represent results obtained from the 2nd hour to the end of the experiment. Compare the upper section with Fig. 5, C, and the lower with Fig. 5, D.

tions. Each animal received 1 gm. of sodium acetoacetate per kilo. Considering only the analyses from the 2nd hour to the end of the experiment, a relation of the β -hydroxybutyric to acetoacetic acid ratio to the concentration of total acetone bodies can be noted similar to that shown by our own data for urine in Fig. 5. The data of Chaikoff and Soskin (Fig. 6) thus show that the equilibrium is established in the body irrespective of the condition of the animal. Fasting, pancreatectomy, and even evisceration do not apparently affect the final equilibrium. It is, however, more readily attained in the intact animals. *This indicates that, although the visceral organs (perhaps chiefly the liver) may be necessary for the rapid conversion of acetoacetic into β -hydroxybutyric acid, the other tissues of the body may bring about the same equilibrium.*

Greater regularity is shown by comparing the β -hydroxybutyric acid content with the content of total acetone bodies. In the lower section of Fig. 6 the calculated β -hydroxybutyric acid content, calculated from the arbitrarily drawn Curve a1 in the upper section, is represented by Curve a2. It will be noted that the β -hydroxybutyric acid content increases regularly as the total acetone body content increases. Striking similarities can be noted when these figures are compared with similar curves representing excretion in urine (Fig. 5, C and D). These data of Chaikoff and Soskin furnish further confirmatory evidence for the statement made above that the conversion of acetoacetate into β -hydroxybutyric acid is related to the unmetabolized portion of the injected salt.

DISCUSSION AND SUMMARY

It is evident that the experimental conditions brought about a considerable change from the normal. The animals were in surgical anesthesia (with amytal). They were injected with strong salt solution, which undoubtedly diluted the blood and affected the water balance of the tissues. The first five dogs received sodium acetoacetate, which resulted in an extreme alkalosis with a very high concentration of NaHCO_3 in the blood. The last six dogs received sodium acetoacetate to which had been added $\frac{1}{2}$ of an equivalent of HCl. The dogs differed in age, weight, and breed. Most of them were fat.

In spite of the abnormal conditions and marked differences in

experimental conditions as noted among animals, the quantity retained (when equilibrium had been attained) was proportional to the rate of injection (Fig. 1). There were considerable differences in tolerance at the same rate in different animals, but these occurred only where solutions of different concentration were used (Fig. 2). The limit of tolerance appears to have been about 5 mm per kilo per hour. The tolerance was not apparently decreased by fasting or pancreatectomy, nor was it increased by insulin or glucose and insulin. This is in agreement with the data of Chaikoff and Soskin.⁵ It was, however, decreased to some extent by fasting and phlorhizin but was restored again to normal by the injection of glucose and insulin. *Looking at the results as a whole, we are impressed by the regularity of the many data from these eleven dogs and the extent of the tolerance over the long injection periods under such apparently abnormal conditions.* These results are the first quantitative data obtained under equilibrium conditions which permit a comparison of the tolerance under various conditions.

Our results also for the first time express in quantitative terms the relation of β -hydroxybutyric acid to acetoacetic acid at various ketosis levels in dogs. From a consideration of the laws of mass action, but without adequate or accurate data, Neubauer (12) states that two factors determine the equilibrium, β -hydroxybutyric acid + O \longleftrightarrow acetoacetic acid + H₂O, namely (a) the concentration of the substances (that is, the grade of acidosis) and (b) the oxygen tension in the tissues. Neubauer found that from 60 to 80 per cent of the acetone bodies excreted by human diabetic subjects was present as β -hydroxybutyric acid. By using the more accurate methods of Shaffer and Marriott, Kennaway (25) was able to show that the β -hydroxybutyric acid of the urine varied from about 32 to about 84 per cent of the total acetone bodies, depending upon the degree of ketosis. This author further pointed out that the quantity of oxygen required to change the equilibrium in a subject from the maximum of β -hydroxybutyric acid to the minimum is small. Thus for the 5th day, in his Case 20, the amount of oxygen required to lower the percentage of β -hydroxy-

⁵ Considering only equilibrium values (Fig. 6), the nephrectomized dogs of Chaikoff and Soskin metabolized acetoacetate at the rate of 1.6 to 2.7 mm per kilo per hour.

butyric acid from 75 to 33 per cent "would be about 280 c.c., an amount which would be absorbed in less than a minute." Although oxygen is necessary to convert β -hydroxybutyric into acetoacetic acid, the oxygen tension alone does not apparently determine the equilibrium. The equilibrium is dependent upon other factors.

Although our ratios from dogs are lower than those found in human subjects with an equal rate of excretion of acetone bodies, they demonstrate a remarkable ability of these animals to reduce *exogenous* acetoacetic acid. The ratio of β -hydroxybutyric to acetoacetic acid increased as the rate of excretion per kilo increased (Fig. 5). At the threshold, the acetone bodies were present largely as acetoacetic acid. The reduction became more efficient as the rate was increased.

It should be noted that our results are expressed per kilo of body weight. It is logical to assume a relation of the rate of excretion to the concentration of total acetone bodies in the blood. Although we have no data from our own animals, such a relation may be inferred from the data of Chaikoff and Soskin. It will be noted that the ratio increased as the total acetone body content of the blood increased. A maximum ratio was soon reached, after which the ratio decreased. *The maximum ratio attained in the blood coincides with the ratio attained in the urine.* The slopes of the curves in Fig. 6 coincide with those in Fig. 5. Although the ratio in the blood is a function of the concentration of the total acetone bodies, such a relation to concentration cannot be demonstrated in the urine. It is more nearly related to the *rate of excretion*.⁶

The reduction of acetoacetic to β -hydroxybutyric acid represents an effective mechanism for minimizing the effect of a very strong acid. The dissociation constant at 25° of acetoacetic acid is 2.6×10^{-4} ; of β -hydroxybutyric acid only 2×10^{-5} (26). It has been suggested that the fatal outcome in diabetes may be due in a measure to a failure to effect the maximum conversion. *It*

⁶ Unpublished data of the author show such a relation also in fasting monkeys and diabetic human subjects. Although the weight differences are great, at the same rate of excretion per kilo of body weight, identical β -hydroxybutyric to acetoacetic acid ratios were attained in the fasting monkeys and human diabetics.

will be noted from Figs. 5 and 6 that the reduction became more efficient as the ketosis increased. It reached a maximum at a blood level of about 9 mM per liter, or at an excretion rate of about 0.8 mM per kilo per hour. This corresponds to an excretion of 1060 mM or cc. of N acid per 24 hours from a 55 kilo human subject. It is interesting to compare this with the highest ketosis found in diabetic human subjects. Thus, Mosenthal and Lewis (27) record a maximum excretion of 1067 mM and Shaffer has noted a maximum excretion of 1153 mM. Friedemann ((7) 1926) found an excretion of approximately 1200 mM (calculated to the same basis) in a fasting capuchin monkey. If the trend is the same in human subjects as in dogs, we may expect the maximum of β -hydroxybutyric acid even at this rate of excretion.

Two distinct processes appear to be involved in the metabolism of acetoacetic acid: (1) oxidation and (2) conversion to β -hydroxybutyric acid. Our data show that the first is not readily affected in the intact animal. It appears to be impaired only slightly in the fasted, phlorhizinized animal. However, this is readily restored to normal by insulin and glucose. But the oxidation is difficult to demonstrate in perfused organs, as can be judged from the experiments of Snapper and Greenbaum (28). Our results, on the other hand, show that the second process, namely the reduction to β -hydroxybutyric acid, is not affected in the intact animal even by fasting and phlorhizin. From the very voluminous literature, it is evident also that the interconversion of acetoacetic to β -hydroxybutyric acid and *vice versa* occurs freely in perfused organs—and even in tissue hashes. *The oxidative mechanism, therefore, appears to be more readily interfered with than the equilibrating mechanism.*

We fail to see that our experiments can give any information on the question of the mode of formation of acetone bodies from fat. We mention this here since most investigators have studied these substances with this in view. Thus, Neubauer and Kennaway, noting the high ratio of β -hydroxybutyric to acetoacetic acid in the urine of diabetics, conclude that β -hydroxybutyric acid is the primary and acetoacetic acid the secondary oxidation product. On the other hand, from a consideration of the rate of metabolism and the ratio of these two acids in the urine after injection or feeding to animals, Blum (13), Dakin (14), Marriott (29),

and Wilder (17) have concluded that acetoacetic acid is the primary oxidation product. This is apparently confirmed by the rate of formation of acetoacetic and β -hydroxybutyric acids in blood following the administration of butyric acid. Thus, Marriott (29) found in the blood of dogs 1.3 and 5.7 mm of total acetone bodies with corresponding β -hydroxybutyric to acetoacetic acid ratios of 0.4 and 3.1 at the 1st and 4th hours, respectively, after injection of sodium butyrate. These ratios are in approximate agreement with those of Chaikoff and Soskin (see Fig. 6) at the same total concentrations. *The ratio, therefore, as our experiments demonstrate, merely represents an equilibrium attained by the excess or unmetabolized portion.* The steps in the metabolism of butyric acid cannot be inferred from such data. The immediate oxidation product of butyric acid may be either β -hydroxybutyric or acetoacetic or crotonic acid (Dakin). The problem is therefore still open.

Of great interest is the relation of the carbohydrate metabolism to the metabolism of the acetone bodies. That carbohydrate plays an important rôle in the prevention of ketosis in the human subject was pointed out by Hirschfeld. Clinical observation has since verified this. The quantitative aspect has been studied by Shaffer (6), Woodyatt (30), Wilder (31), and others. Shaffer has shown that the "ketolytic" ratio of carbohydrate metabolized to fat metabolized in fasting human subjects, in subjects on a high fat diet, and in human diabetics is approximately 1:2. He has called attention to the fact that acetoacetic acid is more reactive chemically than β -hydroxybutyric acid and has described a reaction of acetoacetic acid with glucose. Shaffer and Friedemann (32) have found that 1 mole of glycoaldehyde or glucose will combine with 2 moles of acetoacetate in an oxidative medium (H_2O in 0.5 N alkali). The reaction proceeds also with molecular oxygen, as shown by Shaffer and Harned (5). However, according to Ernst and Förster (33), other substances, non-sugars, including fatty esters themselves, can also be ketolytic. In more recent investigations by Friedemann (34) and Friedemann and Klaas (35), it was found that only the sugars are ketolytic. All sugars studied, from glycoaldehyde to the disaccharoses, bring about the simultaneous oxidation of acetoacetic acid. The ketolytic ratio is the same for all of the sugars; namely, 1 mole of

sugar to 2 moles of acetoacetate. Sucrose, which has no free ose group, is not ketolytic. Only the true sugars are reactive. Substitution in the molecule appears to make the sugar unreactive toward acetoacetate. But, *in vitro* reactions, calculations of the keto-antiketogenic ratios from metabolic data, and the vast mass of clinical data do not offer direct proof of a chemical reaction between metabolizing glucose and the products of fat metabolism. On the other hand, no convincing evidence is at hand which would show that such a reaction cannot and does not occur in the human subject and in monkeys. Except in the case of the fasted, phlorhizinized animals, no definite relation of the plane of carbohydrate metabolism to the metabolism of acetoacetate could be observed in our dogs. Carbohydrate appears to play a definite yet minor rôle. *The high tolerance for acetoacetate in our dogs leads us to the conclusion that intravenously injected sodium acetoacetate is metabolized in these animals by direct oxidation.*

These experiments were undertaken at the suggestion of Professor P. A. Shaffer and carried out with the help of Dr. M. Somogyi and Dr. P. K. Webb. The author wishes to thank them for their help and suggestions generously given throughout this work.

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THE ACTION OF ACID AND ALKALI ON PARATHYROID HORMONE*

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In connection with a previous investigation of the chemical properties of parathyroid hormone (1), we determined the nitrogen partition in the same preparation of parathyroid hormone and correlated the amino nitrogen liberated during gradual acid hydrolysis with the change in potency of the hormone. An account of these later investigations, as well as some further observations on the action of alkali, is included in this paper.

EXPERIMENTAL

Nitrogen Partition in Parathyroid Hormone Preparation—The total nitrogen of the parathyroid hormone preparation, which had been dried to constant weight over sulfuric acid *in vacuo*, was found to be 14.74 per cent by the Koch-McMeekin micro-Kjeldahl method (2). The nitrogen partition found by the Thimann procedure (3) was as follows: acid amide nitrogen 4.39 per cent, humin nitrogen 0.95 per cent, dibasic nitrogen 21.13 per cent, and non-basic nitrogen 71.53 per cent. In several additional analyses substantially the same results were obtained for the first three values of the nitrogen partition, but difficulty was experienced in checking the above value for non-basic nitrogen.

The available peptide nitrogen was determined in the following manner: 50 mg. of the dry hormone preparation were transferred, quantitatively, to a flask and dissolved in 10 cc. of 20 per cent (by weight) hydrochloric acid. The flask was connected with a

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water-cooled reflux condenser by ground glass connections, and placed on a sand bath over an electric hot-plate. 25 hours of continuous boiling were sufficient for complete hydrolysis. After evaporation *in vacuo* to a volume of about 1 cc., the concentrated solution was diluted with water to a volume of 10 cc., and again evaporated to near dryness. The evaporation and addition of water were repeated until most of the free HCl had been removed, after which the solution was transferred, quantitatively, to a 10 cc. volumetric flask and made to volume with water. The amino nitrogen¹ determinations were made on 2 cc. aliquots of this solution in a Van Slyke microapparatus. The available peptide nitrogen was found to constitute 72.5 per cent of the total nitrogen.

Acid Hydrolysis of Parathyroid Hormone—Collip and Clark (4) found that their parathyroid hormone preparation was completely destroyed, or inactivated, after having been boiled in 10 per cent hydrochloric acid for 1 hour. In two preliminary experiments we found that when our preparation was dissolved in 35 per cent (by weight) hydrochloric acid and allowed to stand at room temperature for 1 hour, approximately half of the original potency was retained. However, when the hormone preparation was heated for 1 hour in boiling 10 per cent (by volume) hydrochloric acid, the free amino nitrogen increased from an initial value of 4.53 per cent of the total nitrogen to 33.21 per cent, and simultaneously the hormone appeared to be completely inactivated or destroyed. The results of these experiments indicated the desirability of establishing a closer correlation between the increase in free amino nitrogen and the loss in potency during acid hydrolysis.

Accordingly the hormone preparation was hydrolyzed, gradually, by boiling in 0.05 N hydrochloric acid, and the increase in free amino nitrogen was determined during the course of hydrolysis after the intervals of time shown in Fig. 1. The hormone activity of the untreated protein preparation, which had been standardized as described in the previous paper (1), is represented as 100 per cent at the time hydrolysis began. As hydrolysis proceeded, the loss in potency was estimated by tests in which each of two or more dogs was injected with an amount of the preparation which originally corresponded to 5 units per kilo of body weight and

¹ The free amino nitrogen displaced by nitrous acid within a period of 5 minutes.

should have produced the usual increase of 2 to 8 mg. per cent in the serum calcium within 17 hours if the hormone activity of the protein preparation had been undiminished by the above treatment. When the activity dropped to less than 5 per cent (Fig. 1), two potency tests were made in which the above dosage was doubled. The serum calcium values that were obtained indicated that at least 25 per cent of the original potency was still present. Thus it appears that the close parallelism between the gradual loss in physiological activity and the increase in free amino nitrogen, resulting from hydrolytic cleavage of protein, must be interpreted as additional evidence of the protein nature of parathyroid hormone.

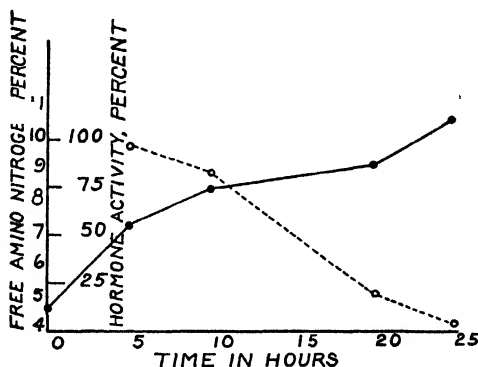


FIG. 1. Hydrolysis of parathyroid hormone. The unbroken line represents the free amino nitrogen expressed as per cent of total nitrogen. The broken line represents the change in per cent of potency produced by gradual acid hydrolysis.

Action of Alkali on Parathyroid Hormone—Very few observations have been reported on the effect of alkali on parathyroid hormone. Collip and Clark (4) stated that their preparation was inactivated by boiling in a 5 per cent solution of sodium hydroxide for 1 hour. Allardyce (5) observed that parathyroid extracts, which were made just alkaline to phenolphthalein, were not inactivated on standing for 4 hours at room temperature. Later Tweedy and Torigoe (6) found that parathyroid hormone was unaffected by 0.08 N alkali after 30 hours at 0–2°. In the experiments which are described below we sought to determine the strength of alkali and the temperature at which gradual inactivation by alkali began.

The hormone preparation which was used in these experiments (Table I) was standardized by tests on twenty different dogs. A definite weight of the hormone preparation per kilo of body weight, when injected subcutaneously, produced an increase in serum calcium of 1.7 to 8 mg. per cent, and an average increase of 5 mg. per cent within 17 hours.

From the data shown in Table I it appears that the hormone is very slowly inactivated by 0.03 N alkali at 0-2°, since an appreciable amount of activity was still present after 246 hours. However, when the strength of the alkali was increased to 0.05 N, and the temperature was maintained at 38° for 5 hours, a biological assay of the hormone showed an average increase in serum calcium

TABLE I

Effect of Sodium Hydroxide on Potency of Parathyroid Hormone Preparation

Experiment No.	Alkali	Time of contact	Temperature	Potency tests	Serum Ca increment		
					Lowest	Highest	Average
	N	hrs.	°C.		mg. per cent	mg. per cent	mg. per cent
1	0.03	24	0-2	2	3.13	7.84	5.84
2	0.03	66	0-2	1			5.71
3	0.03	246	0-2	1			1.61
4	0.03	2	38-39	1			6.22
5	0.03	23	38-39	1	0.20	3.94	0.40
6	0.05	5	38-39	14			1.73

of only 1.73 mg. per cent, or a loss of approximately 67 per cent in the potency.

It seemed likely that inactivation under the above conditions may have been due, at least in part, to racemization. Polariscopic examination of a dilute acid solution of the hormone preparation showed it to be definitely levorotatory, but a brown color immediately developed in the alkaline solution which made it impossible to detect any slight change in optical rotation during the period of contact with alkali. Attempts to remove the colored material by adsorbents resulted in loss of hormone also.

In view of the evidence which indicates the protein nature of parathyroid hormone, we attempted to determine whether inactivation by alkali resulted in the separation of NH_3 from the protein. The following procedure was employed: 200 mg. of

the hormone preparation were dissolved in 15 cc. of 0.05 N sodium hydroxide and held at a temperature of 38–39° for 5 hours while a current of NH_3 -free nitrogen gas was passed through the solution into a flask containing a dilute solution of H_2SO_4 . Nesslerization and colorimetric estimation showed that approximately 0.27 per cent of the total nitrogen was liberated as NH_3 nitrogen.

Thus it appears quite probable that inactivation by alkali may be attributed, at least in part, to disruption of the hormone molecule involving the loss of nitrogen. The source of this nitrogen is probably acid amide groups.

SUMMARY

The total nitrogen value of the parathyroid hormone preparation was 14.74 per cent. The nitrogen distribution was as follows: humin N 0.95 per cent, dibasic N 21.13 per cent, acid amide N 4.39 per cent, and non-basic N 71.53 per cent.

The close parallelism between the increase in free amino nitrogen and the accompanying diminution in hormone activity during gradual acid hydrolysis is additional evidence of the protein nature of parathyroid hormone.

The action of 0.05 N NaOH on the parathyroid hormone preparation during a period of 5 hours at 38° resulted in the liberation of NH_3 and a loss of approximately 67 per cent in potency. It is suggested that the probable source of the NH_3 -N was acid amide groups.

The authors are glad to record their indebtedness to the Committee on Scientific Research, American Medical Association (Grant 328), for financial aid in the purchase of supplies, and technical assistance in the preparation of the hormone. They also wish to thank Swift and Company for their cooperation in supplying glandular material at cost.

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**THE SYNTHESIS OF *d*-ALLOMETHYLOSE BY A SERIES
OF WALDEN INVERSIONS ACCOMPANYING
ALKALINE HYDROLYSIS OF 5-TOSYL
MONOACETONE *l*-RHAMNOSE**

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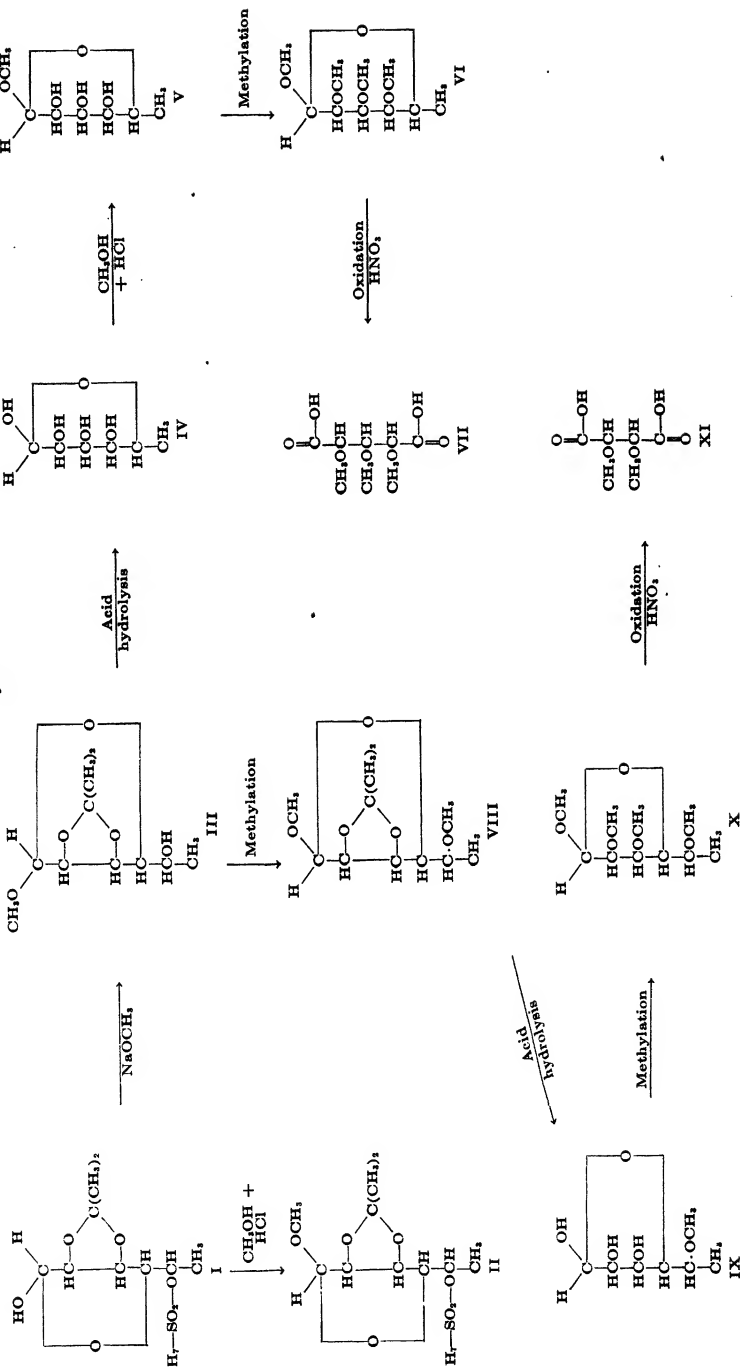
(Received for publication, August 5, 1936)

The phenomenon of Walden inversion is not an infrequent occurrence in sugar chemistry. In recent years it has been observed that Walden inversion was associated with the alkaline detosylation of certain sugar derivatives, the former occurring only under those conditions permitting the intermediate formation of an ethylenic oxide. A novel case of Walden inversion was recently communicated by Hess and Neumann.¹ These authors, on detosylating 2,3,6-trimethyl-4-tosyl-*d*-glucose by means of sodium methylate, obtained 2,3,6-trimethyl-*l*-idose anhydride. In this case there was apparently no free hydroxyl adjacent to the tosyl group, but the Walden inversion took place on the contiguous carbon atom, *i.e.* carbon atom (5). The authors offer no explanation for the mechanism of the reaction. They state, however, that similar treatment of 2,3,6-trimethyl-4-tosyl methylglucoside leads to 2,3,6-trimethyl methylglucoside. Thus, there exists a possibility that also in the case of Hess and Neumann there took place a transitory formation of an ethylenic oxide between carbon atoms (4) and (5), inasmuch as the reaction did not occur when the ring in the <1,5> position was firmly fixed by the glucosidic linkage.

A case to a certain extent analogous but much more complex than that of the above authors is reported in the present communication. This investigation is concerned with the Walden inversions occurring during the alkaline hydrolysis of 5-tosyl

¹ Hess, K., and Neumann, F., *Ber. chem. Ges.*, **68**, 1360 (1935).

Diagram 1



monoacetone *l*-rhamnose. The unimolar tosylation of monoacetone *l*-rhamnose results in the formation of a monotosyl monoacetone *l*-rhamnose derivative (I, Diagram 1). When this compound is treated with anhydrous methyl alcoholic hydrogen chloride at room temperature, 5-tosyl monoacetone methyl-*l*-rhamnofuranoside (II) is formed. Two important conclusions may be drawn from this result. First, the tosyl group in (I) is on carbon atom (5), and, second, no structural changes in the rhamnose molecule occur during tosylation.

The alkaline hydrolysis of (I) with sodium methylate in methyl alcohol results in the formation of a monoacetone methylhexomethyloside (III), which upon acid hydrolysis yields a sugar (IV) quite different from *l*-rhamnose. A comparison of the properties of this sugar with those of *d*-allomethylose² (Table I) shows the striking similarity of the two substances. However, more rigorous proof of the configuration of the new sugar was found by use of the reactions (IV) to (VII) of Diagram 1. Thus, the new sugar (IV) was treated with methyl alcoholic hydrogen chloride to yield β -methyl *d*-allomethyloside (V), which upon complete methylation by the procedure of West and Holden,³ yielded 2,3,4-trimethyl β -methyl *d*-allomethyloside (VI). The completely methylated product upon nitric acid oxidation⁴ suffered cleavage between carbon atoms (5) and (6) to give *i*-trimethoxy-ribo-glutaric acid (VII) from which a crystalline dimethylamide was prepared, m. p. 145–146°. To identify definitely the dimethylamide thus obtained it was necessary to prepare the dimethylamide of *i*-trimethoxy-ribo-glutaric acid not previously obtained from a substance of known structure.⁵ This was accomplished through the nitric acid oxidation of 2,3,4-trimethyl *d*-ribose to yield *i*-trimethoxy-ribo-glutaric acid, whose crystalline dimethylamide

² Mischeel, F., *Ber. chem. Ges.*, **63**, 354 (1930).

³ West, E. S., and Holden, R. F., *J. Am. Chem. Soc.*, **56**, 930 (1934).

⁴ Avery, J., and Hirst, E. L., *J. Chem. Soc.*, 2466 (1929).

⁵ Hibbert, H., and Anderson, C. G., *Canad. J. Research*, **3**, 306 (1930).

By a process of elimination these workers concluded that the dimethylamide derivative (m.p. 145–146°) of an *i*-trimethoxy glutaric acid obtained from the oxidation of completely methylated sedosan was of the ribose structure, but no direct verification was made. The results obtained in the present work substantiate the conclusion that this substance was the dimethylamide of *i*-trimethoxy-ribo-glutaric acid.

melted at 145–146°. The analyses, optical inactivity, melting points, and mixed melting point of this material with the dimethylamide of the *i*-trimethoxy glutaric acid obtained from the new sugar proved that the two substances were identical.

The oxidation of (VI) to yield (VII) proves conclusively that the configurations of carbon atoms (2), (3), and (4) are the same as that of ribose. Depending upon the configuration of carbon atom (5), however, two hexomethyloses could give rise to *i*-trimethoxy-ribo-glutaric acid, namely *d*-allomethylose and *l*-talomethylose. The physical constants of *l*-talomethylose⁶ and its phenylosazone derivative are at such variance with those of the new sugar

TABLE I

Comparison of Properties of the Sugar Obtained by Inversion of l-Rhamnose with d-Allomethylose and l-Talomethylose

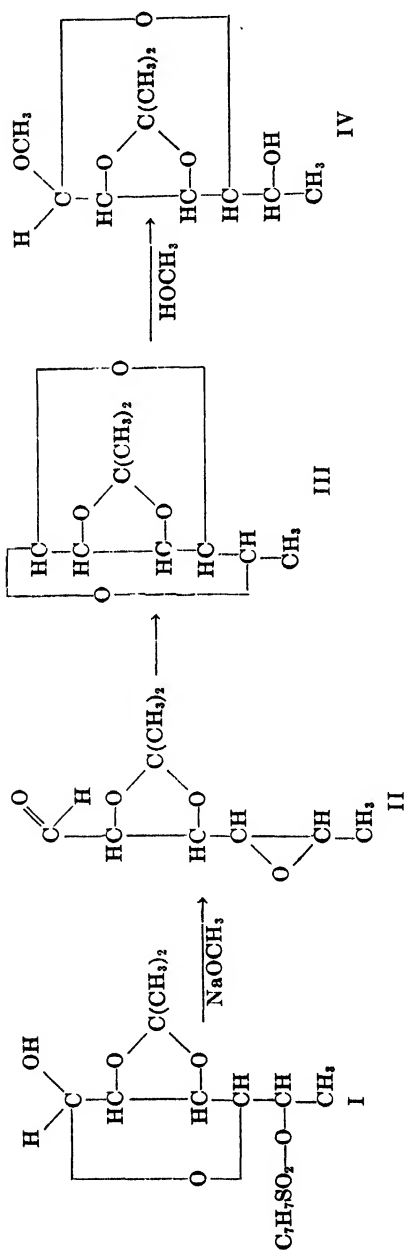
M. p., °C.			[α] _D , degrees		
New Sugar	<i>d</i> -Allo-methylose	<i>l</i> -Talo-methylose	New Sugar	<i>d</i> -Allomethylose	<i>l</i> -Talomethylose
Hexomethyloses					
151–152	146	Sirup	−8.5 → +1.2	−12 → −1.0	−9.0 (Constant)
Hexomethylose phenylosazones					
184–185	182–183	178	−79.1 → −49.9	−72.3 → (?)	

(Table I) as to eliminate it as a possibility. Hence, it follows that the new sugar is *d*-allomethylose.

Before the mechanism of the Walden inversion accompanying the alkaline hydrolysis of 5-tosyl monoacetone *l*-rhamnose to yield *d*-allomethylose can be adequately treated, it is necessary to determine the ring structure of the monoacetone methyl-*d*-allomethyloside directly obtained. Two methods were successfully used: first, comparing the rate of acid hydrolysis of this product with monoacetone methyl-*l*-rhamnofuranoside (Table II), and second, the series of reactions (VIII) to (XI) of Diagram 1. The first method showed that the ring structure of (III) was most

⁶ Votoček, E., and Červany, J., *Ber. chem. Ges.*, **48**, 658 (1915).

Diagram 2



likely the same as that of the rhamnofuranose derivative and the furanose ring structure was confirmed by methylation and oxidation methods. Thus, methylation of (III) yielded 5-methyl monoacetone methyl-*d*-allomethyloside (VIII), which upon acid hydrolysis gave sirupy 5-methyl *d*-allomethylose (IX). This mono-methyl allomethylose when treated with methyl alcoholic hydrogen chloride yielded 5-methyl methyl-*d*-allomethyloside and upon complete methylation 2,3,5-trimethyl methyl-*d*-allomethyloside (X). Upon oxidation with nitric acid, this product gave *i*-dimethoxy succinic acid (XI), identified by conversion to the crystalline dimethylamide,⁷ m. p. 208–209°. Had the ring structure of (III) been <1,5>, *i*-trimethoxy-ribo-glutaric acid would have been obtained. The furanose <1,4> ring structure of (III) is thus definitely shown.

A rigorous analysis of the mechanism of the double inversion here described is at this stage of our knowledge impossible. It may be permissible, however, to speculate that in the present case a transitory aldehydo 2,3-monoacetone<4,5> anhydro-*l*-rhamnose (II, Diagram 2) is formed which passes into monoacetone methyl-*d*-allomethylofuranoside (IV). By analogy with the case of Hess and Neumann, a transitory 2,3-monoacetone <1,5> anhydro-hexamethylose (III) is also probably formed as the second phase of the transformation. The succession of Walden inversions cannot be definitely ascertained.

EXPERIMENTAL

5-Tosyl Monoacetone l-Rhamnose—Monoacetone β -*l*-rhamnose (5 gm.) (m.p. 93–94°) was dissolved in 25 cc. of dry pyridine and 4.81 gm. (1.1 moles) of *p*-toluene sulfonyl chloride dissolved in 10 cc. of chloroform were added with stirring at 0°. The solution was then allowed to stand in the ice bath for 1 hour, followed by a period of 12 hours at room temperature. Water (1 cc.) was then added, and the mixture thoroughly shaken and allowed to stand 30 minutes. The solution was then diluted with chloroform, washed twice with water, twice with ice-cold 10 per cent sulfuric acid solution, twice with ice-cold saturated sodium bicarbonate solution, and finally twice with ice water. The chloroform extract, after drying over calcium chloride, was concentrated under

⁷ Goodyear, E. H., and Haworth, W. N., *J. Chem. Soc.*, 3144 (1927).

diminished pressure at 40° to a thick sirup from which the last traces of chloroform were removed by dissolving in dry toluene and concentrating as before. The sirup was then taken up in dry toluene and petroleum ether (30–40°) was added till a slight turbidity persisted. Crystallization soon began, and, after standing in the cold overnight, the product was removed by filtration. Yield 3.5 gm. After a second recrystallization, a melting point of 92–93° was obtained, unchanged by further recrystallizations. The specific rotation of the substance in U.S.P. chloroform was

$$[\alpha]_D^{24} = \frac{+1.76^\circ \times 100}{2 \times 3.026} = +29.1^\circ$$

The composition of the substance agreed with that of a monotosyl monoacetone hexomethylose.

3.809 mg. substance: 7.425 mg. CO₂ and 2.150 mg. H₂O

6.780 " " : 4.550 " BaSO₄

C₁₆H₂₂O₇S. Calculated. C 53.59, H 6.14, S 8.93

358.3 Found. " 53.29, " 6.34, " 9.22

The substance is very sensitive to both acid and alkali but is very stable when kept under anhydrous conditions. The substance is very soluble in acetone, alcohols, benzene, and toluene, slightly soluble in ether, and insoluble in light petroleum ether and water.

Preparation of 5-Tosyl Monoacetone Methyl-l-Rhamnofuranoside from 5-Tosyl Monoacetone l-Rhamnose—5-Tosyl monoacetone rhamnose (2.0042 gm.) was dissolved in exactly 20 cc. of anhydrous methyl alcohol containing 0.5 per cent of dry hydrogen chloride. The initial specific rotation, $[\alpha]_D^{24} = +34.1^\circ$, had changed at the end of 3 days at room temperature to the constant value $[\alpha]_D^{24} = -14.7^\circ$. The acid was then neutralized with silver carbonate and the filtered solution diluted to 200 cc. with ice water. After standing for a few minutes, crystallization began and the product was removed by filtration. Yield 1.8 gm. After one recrystallization from methyl alcohol the substance gave a constant melting point of 83–84°, which remained unchanged when mixed with an authentic specimen of 5-tosyl monoacetone methylrhamnofuranoside. The specific rotation in methyl alcohol was

$$[\alpha]_D^{25} = \frac{-0.86^\circ \times 100}{2 \times 3.010} = -14.28^\circ$$

Inversion of 5-Tosyl Monoacetone l-Rhamnose with Sodium Methylate to Yield Monoacetone Methyl-d-Allomethylofuranoside—5-Tosyl monoacetone l-rhamnose (8.0 gm.) was dissolved in 100 cc. of absolute methyl alcohol containing 2.35 gm. of sodium methylate (2 moles) and allowed to stand at room temperature overnight. The reaction began after a few minutes, as was shown by the separation of sodium *p*-toluene sulfonate and was quantitatively complete after 12 hours. The excess sodium methylate was then decomposed with carbon dioxide and the solution concentrated under diminished pressure to a dry mass. The product was then removed by the repeated extraction of the salts with dry ether. After drying over calcium chloride, the ether extract was filtered and concentrated under diminished pressure to a sirup which distilled completely at 68–70° at 0.2 mm. Yield 4.0 gm. After standing in the cold for some time, the substance crystallized, m.p. 22°. The specific rotation of the substance in absolute methyl alcohol was

$$[\alpha]_D^{25} = \frac{-6.00^\circ \times 100}{2 \times 4.042} = -74.2^\circ$$

The composition of the substance agreed with that of a monoacetone methylhexomethyloside.

4.600 mg. substance: 9.300 mg. CO₂ and 3.470 mg. H₂O

6.430 " " : 6.810 " AgI

C₁₆H₁₈O₆. Calculated. C 55.02, H 8.3, OCH₃ 14.22

218.1 Found. " 55.13, " 8.4, " 13.98

The substance does not reduce warm Fehling's solution, but after hydrolysis with dilute acid it reduces strongly. The substance is soluble in all the usual organic solvents but is only slightly soluble in cold water.

5-Tosyl Monoacetone Methyl-d-Allomethylofuranoside—Monoacetone methyl-d-allomethylofuranoside (1.0 gm.) was dissolved in 1.5 cc. of dry pyridine and 1.6 gm. of tosyl chloride added. After shaking for a few minutes, the solution was allowed to stand overnight at room temperature. A small amount of water was then added, and the solution was shaken and allowed to stand 30 minutes. The mixture was then diluted with saturated sodium bicarbonate solution to about 10 cc., whereupon the oily layer first separating crystallized completely. The product was re-

moved by filtration, thoroughly washed with water, and air-dried at room temperature. Yield 1.8 gm. After one recrystallization from absolute methyl alcohol a constant melting point of 93–94° was obtained. The specific rotation of the substance in methyl alcohol was

$$[\alpha]_D^{25} = \frac{-3.50^\circ \times 100}{2 \times 3.738} = -46.8^\circ$$

The analysis of the substance agreed with that of a tosyl monoacetone methylhexomethyloside.

4.400 mg. substance:	8.815 mg. CO ₂ and 2.620 mg. H ₂ O
7.915 " " "	: 5.240 " AgI
C ₁₇ H ₂₄ O ₇ S. Calculated.	C 54.80, H 6.45, OCH ₃ 8.32
372.26 Found.	" 54.62, " 6.66, " 8.73

The substance is soluble in methyl alcohol, ethyl alcohol, and benzene, slightly soluble in ether, and insoluble in petroleum ether and water.

Reductive Hydrolysis of 5-Tosyl Monoacetone Methyl-d-Allomethyloside to Yield Pure Monoacetone Methyl-d-Allomethyloside—10 gm. of 5-tosyl monoacetone methyl-*d*-allomethyloside (m.p. 93–94°) were dissolved in 500 cc. of 80 per cent methyl alcohol and 125 gm. of 4 per cent sodium-mercury amalgam added in small portions over a period of 1 hour. The reaction was allowed to proceed with stirring until the amalgam was completely decomposed, which was usually accomplished in 12 to 14 hours. The solution was then filtered, the excess alkali neutralized by means of a stream of carbon dioxide gas, and the solution concentrated to dryness under diminished pressure at 40°. The resulting salts were rendered practically anhydrous by the addition of absolute ethyl alcohol, followed by distillation under diminished pressure. The solid mass was then repeatedly extracted with hot ether, and the ether extract filtered, dried over sodium sulfate, filtered, and concentrated to a thick sirup under diminished pressure. The product distilled completely under diminished pressure, b.p. 85–86° at 0.3 mm. Yield 5.0 gm. The specific rotation of the substance purified in this manner in methyl alcohol was

$$[\alpha]_D^{25} = \frac{-5.17^\circ \times 100}{2 \times 3.308} = -78.1^\circ$$

The composition of the substance agreed with that of a monoacetone methylhexomethyloside.

5.840 mg. substance: 11.800 mg. CO₂ and 4.380 mg. H₂O

6.930 " " : 7.390 " AgI

C₁₀H₁₈O₆. Calculated. C 55.02, H 8.3, OCH₃ 14.22

218.1 Found. " 55.10, " 8.39, " 14.07

To make certain that the purification process described above caused no change in the structure of the molecule, the following experiment was carried out. 0.5 gm. of the purified monoacetone methyl-*d*-allomethyloside was dissolved in 1 cc. of pyridine and 0.9 gm. of tosyl chloride added. The product obtained (0.7 gm.) was identical with the original 5-tosyl monoacetone methyl-*d*-allomethyloside as determined by a melting point of 93–94° and specific rotation in methyl alcohol.

$$[\alpha]_D^{25} = \frac{-2.71^\circ \times 100}{2 \times 3.00} = -45.16^\circ$$

This method of purifying the crude monoacetone methyl-*d*-allomethyloside was therefore employed in obtaining the pure product.

d-Allomethylose—14.0 gm. of pure monoacetone methyl-*d*-allomethyloside were dissolved in 300 cc. of 1.5 per cent sulfuric acid solution and heated at 100° for 60 minutes. The acid was then neutralized with excess barium carbonate. The solution was filtered and concentrated under diminished pressure at 40° to a thick sirup. Upon the addition of ethyl alcohol, crystallization began and the product was removed by filtration. Yield 9.5 gm. After a third crystallization from ethyl alcohol, a melting point of 151–152° was obtained, which was unchanged by further recrystallizations. The initial specific rotation in water

$$[\alpha]_D^{25} = \frac{-0.69^\circ \times 100}{2 \times 4.034} = -8.5^\circ \text{ (4 minutes)}$$

changed in 35 minutes to the constant value

$$[\alpha]_D^{25} = \frac{+0.10^\circ \times 100}{2 \times 4.034} = +1.2^\circ$$

The composition of the substance agreed with that of a hexomethylose.

4.806 mg. substance: 7.770 mg. CO₂ and 3.200 mg. H₂O

C₆H₁₀O₆. Calculated. C 43.90, H 7.31
164.0 Found. " 44.08, " 7.45

The substance is soluble in pyridine, methyl alcohol, ethyl alcohol, and water; insoluble in ether, petroleum ether, and benzene. It reduces Fehling's solution readily upon warming and has a very sweet taste.

The reducing property of the sugar in alkaline hypiodite solution is very similar to that of *l*-rhamnose. Thus, 64.4 mg. of *d*-allomethylose consumed 6.52 cc. of 0.1 N iodine, whereas 64.8 mg. of *l*-rhamnose consumed 6.56 cc. of 0.1 N iodine solution.

d-Allomethylose *p*-Bromophenylhydrazone—0.5 gm. of *d*-allomethylose was dissolved in 15 cc. of hot ethyl alcohol and 0.5 gm. (1 mole) of *p*-bromophenylhydrazine dissolved in 5 cc. of ethyl alcohol added. The mixture was heated on the water bath for 10 minutes and the alcohol allowed to evaporate. The sirup remaining crystallized upon cooling and was recrystallized from ether-petroleum ether to the constant melting point of 145–146°, with sintering at 140°. Yield 0.6 gm. The specific rotation of the substance in dry pyridine was

$$[\alpha]_D^{25} = \frac{-0.52^\circ \times 100}{1 \times 2.368} = -21.9^\circ$$

changing after 24 hours to the constant value

$$[\alpha]_D^{25} = \frac{-0.28^\circ \times 100}{1 \times 2.368} = -11.8^\circ$$

The composition of the substance agreed with that of a hexomethylose bromophenylhydrazone.

4.012 mg. substance: 6.375 mg. CO₂ and 1.890 mg. H₂O

5.890 " " : 0.430 cc. N₂ (23° and 772 mm.)

C₁₂H₁₇O₄N₂Br. Calculated. C 43.24, H 5.14, N 8.40
333.06 Found. " 43.33, " 5.26, " 8.55

d-Allomethylose Phenylsazone—0.2 gm. of *d*-allomethylose (m.p. 151–152°) was dissolved in 10 cc. of water and 0.4 gm. of phenylhy-

drazine (3 moles) dissolved in 2 cc. of glacial acetic acid added. The solution was heated on the boiling water bath for 15 minutes, whereupon the osazone began to crystallize. After cooling, the product was removed by filtration and washed thoroughly with water. The yellow crystalline mass was then triturated with methyl alcohol, filtered, and washed with methyl alcohol. The melting point of the dried needles was 184–185°, unchanged by further recrystallizations from 50 per cent methyl alcohol or triturating with absolute methyl alcohol. The specific rotation of the substance in pyridine-ethyl alcohol (3:2) was

$$[\alpha]_D^{25} = \frac{-0.88^\circ \times 100}{1 \times 1.112} = -79.1^\circ$$

changing after 8 days to the constant value

$$[\alpha]_D^{25} = \frac{-0.55^\circ \times 100}{1 \times 1.112} = -49.4^\circ$$

The composition of the substance agreed with that of a hexomethylose phenylosazone.

4.185 mg. substance: 9.657 mg. CO₂ and 2.400 mg. H₂O

4.120 " " : 0.594 cc. N₂ (26° and 763 mm.)

C₁₈H₂₈O₈N₄. Calculated. C 62.97, H 6.73, N 16.30

343.0 Found. " 62.92, " 6.41, " 16.52

α- and *β*-Methyl-*d*-Allomethylpyranoside—2.0 gm. of *d*-allo-methylose (m.p. 151–152°) were dissolved in 50 cc. of methyl alcohol containing 2 per cent of dry hydrogen chloride and heated under the reflux for 2 hours. The solution was then cooled, the acid neutralized with an excess of silver carbonate, and the solution filtered and concentrated under diminished pressure at 40° to a thick sirup. The product was dissolved in ethyl acetate, treated with charcoal, and filtered. Upon cooling, the *β*-methylallo-methylloside crystallized from solution. Yield 1.4 gm. After a second recrystallization the melting point of 94–95° was obtained, which was unchanged by further recrystallization.

The specific rotation of the *β*-methylglycoside in water was

$$[\alpha]_D^{25} = \frac{-2.48^\circ \times 100}{2 \times 2.024} = -61.26^\circ$$

The ease with which the β -methylglycoside crystallizes from ethyl acetate makes its separation from the sirupy α -methylglycoside possible. The mother liquors from the β isomer upon concentration yielded a sirup which distilled under a high vacuum. B.p. 105–106° at 0.3 mm. Yield 0.6 gm. The specific rotation of the practically pure α -methylglycoside in water was

$$[\alpha]_D^{25} = \frac{+1.11^\circ \times 100}{1 \times 2.048} = +54.2^\circ$$

The composition of both the α and β isomers agreed with that of a methylhexomethyloside.

3.725 mg.	β isomer:	6.475 mg.	CO ₂	and	2.615 mg.	H ₂ O
4.730	" " "	:	15.684 cc.	0.01 N	Na ₂ S ₂ O ₃	
3.805	" " "	:	12.740 "	0.01	" "	
	C ₇ H ₁₄ O ₆ .	Calculated.	C 47.16,	H 7.92,	OCH ₃ 17.40	
178.1		Found.				
	β isomer.	"	47.40,	" 7.85,	" 17.14	
	α "	"		"	17.30	

Neither the α - nor β -methylglycoside reduces warm Fehling's solution, but after acid hydrolysis, both give a strong reduction. The α isomer is more soluble in the usual organic solvents than the β isomer, such as ethyl acetate, methyl alcohol, ethyl alcohol, and pyridine. Both forms are very soluble in water and pyridine.

2,3,4-Trimethyl β -Methyl-d-Allomethylpyranoside—1.0 gm. of β -methylallomethyloside (m.p. 93°) was dissolved in 2 cc. of water, and 5 cc. of carbon tetrachloride containing 3.6 cc. of dimethyl sulfate were added. The temperature was then carried to 55° and under vigorous stirring 16 cc. of 60 per cent sodium hydroxide solution were added according to the procedure of West and Holden.³ The temperature was then raised to 70–75° and 6.4 cc. of dimethyl sulfate added, after which the temperature was maintained at 100° for 30 minutes. The solution was then cooled, sufficient water was added to dissolve the salts, and it was thoroughly extracted with chloroform. The chloroform extract was then washed with water, dried over anhydrous sodium sulfate, filtered, and concentrated under diminished pressure at 40° to a thick sirup which distilled completely under a high vacuum. B.p. 60–65° at 0.3 mm.

The product after a second methylation in the manner described

was completely methylated. B.p. 60–62° at 0.3 mm. Yield 0.8 gm. $n_D^{21} = 1.4451$. The specific rotation of the substance in water was

$$[\alpha]_D^{25} = \frac{-1.85^\circ \times 100}{2 \times 2.126} = -43.5^\circ$$

The composition of the substance agreed with that of a trimethyl methylhexomethylloside.

3.355 mg. substance:	6.695 mg. CO ₂ and 2.730 mg. H ₂ O
3.095 " " :	13.130 " AgI
C ₁₀ H ₂₀ O ₆ .	Calculated. C 54.49, H 9.15, OCH ₃ 56.31
220.2	Found. " 54.41, " 9.10, " 56.00

The substance is soluble in all the usual organic solvents but is only slightly soluble in cold water.

Preparation of the Dimethylamide of i-Trimethoxy-Ribo-Glutaric Acid from 2,3,4-Trimethyl β-Methyl-d-Allomethylloside—0.6 gm. of 2,3,4-trimethyl β-methylallomethylloside was dissolved in 6 cc. of nitric acid (*d* 1.42) and allowed to stand 1 hour at room temperature. The solution was then heated gradually to 70° and allowed to remain at this temperature for 15 minutes, after which a temperature of 95° was maintained for 2 hours. The solution was then concentrated under a high vacuum to a thin sirup from which the last traces of nitric acid and water were removed by the repeated addition of absolute ethyl alcohol and dry benzene, followed by distillation under diminished pressure. The sirup was then dissolved in 25 cc. of methyl alcohol containing 2 per cent of dry hydrogen chloride and refluxed for 6 hours on the steam bath. The acid was then neutralized with an excess of silver carbonate, and the solution filtered and concentrated under diminished pressure to a thick sirup which distilled under a high vacuum at 110–112° (bath temperature) under 0.3 mm. pressure. Yield 0.3 gm. $n_D^{22} = 1.4352$. The composition of the substance agreed with that of a dimethyl ester of trimethoxyglutaric acid.

5.502 mg. substance:	9.670 mg. CO ₂ and 3.500 mg. H ₂ O
3.416 " " :	16.000 " AgI
C ₁₀ H ₁₈ O ₇ .	Calculated. C 47.98, H 7.20, OCH ₃ 61.97
250.1	Found. " 47.92, " 7.11, " 61.82

0.3 gm. of the methyl ester of *i*-trimethoxy-ribo-glutaric acid prepared as above was dissolved in 5 cc. of methyl alcohol and saturated at 0° with dry methylamine. The solution was allowed to stand 2 days at 15°, after which it was concentrated to dryness under diminished pressure at 40°. The sirup thus obtained soon crystallized and after recrystallizing from ethyl acetate, gave a melting point of 145–146°, unchanged by further recrystallization. A mixed melting point of this material with an authentic specimen of the dimethylamide of trimethoxy-ribo-glutaric acid showed no depression. The substance when dissolved in methyl alcohol (*c* 3.148) showed no optical activity. The analysis of the substance agreed with that of a dimethylamide of a trimethoxy-glutaric acid.

4.799 mg. substance: 8.500 mg. CO₂ and 3.495 mg. H₂O

5.600 " " : 0.550 cc. N₂ (24° and 762.5 mm.)

C₁₀H₂₀O₅N₂. Calculated. C 48.35, H 8.12, N 11.28

248.16 Found. " 48.30, " 8.14, " 11.32

Preparation of the Dimethylamide of i-Trimethoxy-Ribo-Glutaric Acid from 2,3,4-Trimethyl-d-Ribose—In a manner exactly analogous to that described above for the preparation of the dimethylamide of *i*-trimethoxy-ribo-glutaric acid from 2,3,4-trimethyl-β-methylallomethyloside, the dimethylamide of trimethoxy-ribo-glutaric acid (0.25 gm.) was prepared from 2,3,4-trimethyl ribose (0.35 gm.).⁸ The product obtained in this manner gave a melting point of 145–146° after one recrystallization from ethyl acetate which was unchanged by further recrystallizations. A 2 per cent solution of this substance in methyl alcohol showed no optical activity. The analysis of the substance agreed with that of a dimethylamide of trimethoxyglutaric acid.

4.305 mg. substance: 7.675 mg. CO₂ and 3.130 mg. H₂O

4.130 " " : 0.407 cc. N₂ (28° and 761 mm.)

C₁₀H₂₀O₅N₂. Calculated. C 48.35, H 8.12, N 11.28

248.16 Found. " 48.61, " 8.14, " 11.18

⁸ A sample of 2,3,4-trimethylribose carefully purified by Dr. R. S. Tipson of this laboratory, with a melting point of 98–100°, was used in this experiment. With the consent of this worker the melting point originally given by Levene and Tipson (Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **93**, 623 (1931)) is corrected at this time.

• *Ring Structure of Monoacetone Methyl-d-Allomethyloside*

(a) *Acid Hydrolysis of Monoacetone Methyl-d-Allomethyloside—*

The hydrolysis of monoacetone methyl-d-allomethyloside with 0.03 N hydrochloric acid at 100° was carried out by the procedure previously reported by Levene and Compton⁹ for monoacetone methyl-l-rhamnofuranoside. In this experiment 0.0743 gm. of monoacetone methyl-d-allomethyloside was dissolved in 5.0 cc. of 0.03 N HCl. A comparison of the rate of hydrolysis of this material with that of monoacetone methyl-l-rhamnose at 100° shows the striking similarity of the two substances (Table II).

TABLE II

Rate of Hydrolysis of Monoacetone Methyl-d-Allomethyloside and Monoacetone Methyl-l-Rhamnofuranoside with 0.03 N HCl at 100°

Time	[α] _D ²⁰		Per cent completion	
	Monoacetone methyl-d-allomethyloside	Monoacetone methyl-l-rhamnofuranoside	Monoacetone methyl-d-allomethyloside	Monoacetone methyl-l-rhamnofuranoside
<i>min.</i>	<i>degrees</i>	<i>degrees</i>		
0	-66.6	-58.1	0	0
5	-30.3	-50.4	53.7	12.3
10	-9.1		85.0	
15	-1.0	-5.0	97.2	85.0
20	+1.0 (Constant)		100.0	
25		+3.6		97.1
30		+5.4 (Constant)		100.0

(b) *Structure Based on Methylation and Oxidation Methods.*

5-Methyl Monoacetone Methyl-d-Allomethyloside—4.0 gm. of pure monoacetone methyl-d-allomethyloside were subjected to six methylations with Purdie's reagent (10 gm. of silver oxide and 10 cc. of methyl iodide at 50° with good stirring for 24 hours were used in each methylation). Final yield, 2.5 gm. B.p: 85–86° at 0.3 mm. The specific rotation of the substance in methyl alcohol was

$$[\alpha]_D^{20} = \frac{-3.32^\circ \times 100}{2 \times 2.118} = -78.4^\circ$$

⁹ Levene, P. A., and Compton, J., *J. Am. Chem. Soc.*, **57**, 2306 (1935).

The composition of the substance agreed closely with that of a methyl monoacetone methylhexomethyloside.

4.410 mg. substance: 9.090 mg. CO₂ and 3.370 mg. H₂O

5.200 " " : 26.30 cc. 0.01 N Na₂S₂O₃

C₁₁H₂₀O₅. Calculated. C 56.86, H 8.70, OCH₃ 26.72

232.2 Found. " 56.20, " 8.55, " 26.03

5-Methyl d-Allomethylose—2.5 gm. of 5-methyl monoacetone methyl-*d*-allomethyloside were hydrolyzed with 50 cc. of 1 per cent sulfuric acid solution by heating for 2 hours at 100°. The solution was then cooled and the acid neutralized with an excess of barium carbonate, treated with charcoal, and filtered. The clear solution was then concentrated under diminished pressure at 40° to a thick sirup which was dried by dissolving in absolute ethyl alcohol, adding dry benzene, and concentrating under diminished pressure. After this process had been repeated several times, the product was dissolved in a small volume of ethyl alcohol and the solution was filtered, diluted with a large volume of dry ether, again filtered, and concentrated under diminished pressure to a thick sirup which could not be induced to crystallize. Yield 1.2 gm. The analysis of the substance agreed with that of a methyl hexomethylose.

4.636 mg. substance: 7.995 mg. CO₂ and 3.25 mg. H₂O

4.121 " " : 13.84 cc. 0.01 N Na₂S₂O₃

C₇H₁₄O₅. Calculated. C 47.16, H 7.92, OCH₃ 17.40

178.1 Found. " 47.02, " 7.84, " 17.31

2,3,5-Trimethyl Methyl-d-Allomethyloside—1.2 gm. of 5-methyl-*d*-allomethylose were dissolved in 20 cc. of methyl alcohol containing 1 per cent of dry hydrogen chloride and allowed to stand 24 hours at room temperature. The solution was then heated at 50° for 2 hours, after which it gave only a very feeble reduction with Fehling's solution. The solution was then cooled, the acid neutralized with an excess of silver carbonate, filtered, and concentrated under diminished pressure at 40° to a thick sirup. The product was dissolved in dry ether, and the ethereal solution was filtered, dried over anhydrous sodium sulfate, filtered, and concentrated under diminished pressure to a thick sirup which distilled under a high vacuum. B.p. 105–110° at 0.3 mm. Yield

0.8 gm. The substance was not further characterized but its properties corresponded with those expected for a methyl methylhexomethyloside.

The product obtained above was subjected to two methylations with dimethyl sulfate (10 cc.) in the presence of 60 per cent sodium hydroxide solution (16 cc.) and 5 cc. of carbon tetrachloride, according to the procedure of West and Holden.³ There was thus obtained 0.55 gm. of substance with a boiling point of 78–80° at 0.3 mm. The analysis of the substance agreed with that of a trimethyl methylhexomethyloside.

3.042 mg. substance: 6.0905 mg. CO₂ and 2.562 mg. H₂O

3.190 " " : 13.580 " AgI

C₁₀H₂₀O₅. Calculated. C 54.49, H 9.15, OCH₃, 56.31

220.2 Found. " 54.59, " 9.42, " 56.18

Preparation of the Dimethylamide of i-Dimethoxy Succinic Acid from 2,3,5-Trimethyl Methyl-d-Allomethyloside—0.55 gm. of 2,3,5-trimethyl methyl-d-allomethyloside was dissolved in 5.5 cc. of nitric acid (*d* 1.42) and allowed to stand 1 hour at room temperature. The mixture was then gradually heated to a temperature of 95–100° which was then maintained for 2 hours. The method of isolating and esterifying the *i*-dimethoxy succinic acid to obtain the dimethyl ester of *i*-dimethoxy succinic acid was similar to that described above for the preparation of the dimethyl ester of *i*-trimethoxy-ribo-glutaric acid. Yield 0.15 gm. B.p. (bath temperature) 112–115°. No attempt was made to crystallize this product, since it was contaminated with other oxidation products.

The product obtained above consisting largely of the dimethyl ester of *i*-dimethoxy succinic acid (0.15 gm.) was dissolved in 1.5 cc. of methyl alcohol and saturated at 0° with dry methylamine. After standing at 15° for 2 days the solution was concentrated to dryness under diminished pressure. The crude product thus obtained gave a melting point of 208–209° after one recrystallization from ethyl acetate. The yield was about 50 mg. A mixed melting point of this material with an authentic specimen¹⁰ of the

¹⁰ The reference compound was prepared from a sample of the dimethyl ester of *i*-dimethoxy succinic acid prepared by Dr. R. S. Tipson of this laboratory.

dimethylamide of *i*-dimethoxy succinic acid showed no depression (m.p. 208–209°). The analysis of the substance agreed with that of a dimethylamide of a dimethoxy succinic acid.

4.488 mg. substance: 7.780 mg. CO₂ and 3.175 mg. H₂O

2.810 “ “ : 0.343 cc. N₂ (28° and 758 mm.)

C₈H₁₆O₄N₂. Calculated. C 47.03, H 7.89, N 13.71

204.1 Found. “ 47.27, “ 7.93, “ 13.75

A NEW METHOD FOR THE PREPARATION OF FURANOSE DERIVATIVES OF PENTOSE. MONOACETONE *l*-ARABOFURANOSIDE

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A study of the order of reactivity of the hydroxyl groups in certain derivatives of monosaccharides toward acylating reagents has shown that the primary hydroxyl is preferentially esterified. Making use of the open chain mercaptal derivative of hexoses, Lieser and Schweizer¹ found that unimolar benzylation always resulted in the formation of a 6-benzoyl derivative. Similarly, with the pentoses, monobenzoyl pentose mercaptal derivatives were obtained in which the benzoyl group was thought to be in position (5). Direct experimental evidence in favor of this assumption, however, was lacking.

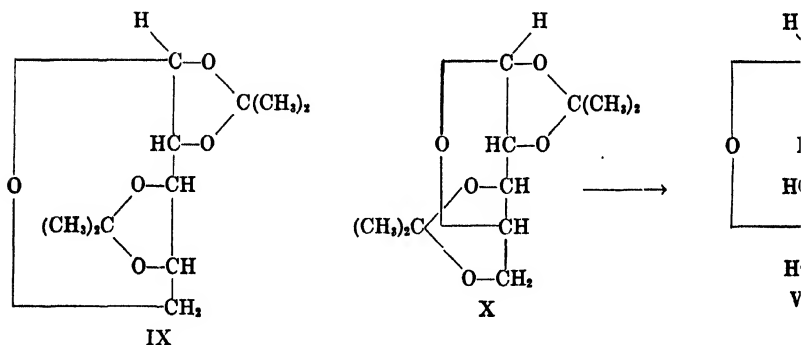
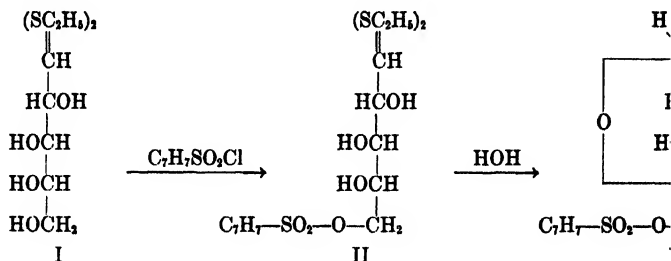
Definite information as to the location of the acyl group was of great importance in connection with the work that has been in progress in this laboratory for some time, directed toward the synthesis of furanose derivatives of pentoses. For the solution of this problem, advantage was taken of the unusual stability of *p*-toluenesulfonyl (tosyl) derivatives of sugars. Thus *l*-arabinose diethylmercaptal (I) when treated with 1 equivalent of *p*-toluenesulfonyl chloride in pyridine solution yielded a crystalline tosyl *l*-arabinose diethylmercaptal (II). The position of the tosyl group has been determined by two independent methods which show conclusively that the tosyl group is in position (5).

The removal of the mercaptal groups from (II) may be easily accomplished by use of the method developed by Wolfrom² for the preparation of aldehydo-sugar acetates, to yield 5-tosyl

¹ Lieser, T., and Schweizer, R., *Ann. Chem.*, **519**, 271 (1935).

² Wolfrom, M. L., *J. Am. Chem. Soc.*, **51**, 2188 (1929); **52**, 2464 (1930).

l-arabinose (III). In this substance the oxidic ring can only occupy the <1,4> positions and this general method of preparing furanose derivatives of pentoses should be capable of wide extension. When an aqueous solution of (III) is treated with phenylhydrazine in acetic acid solution, a crystalline hydrazone

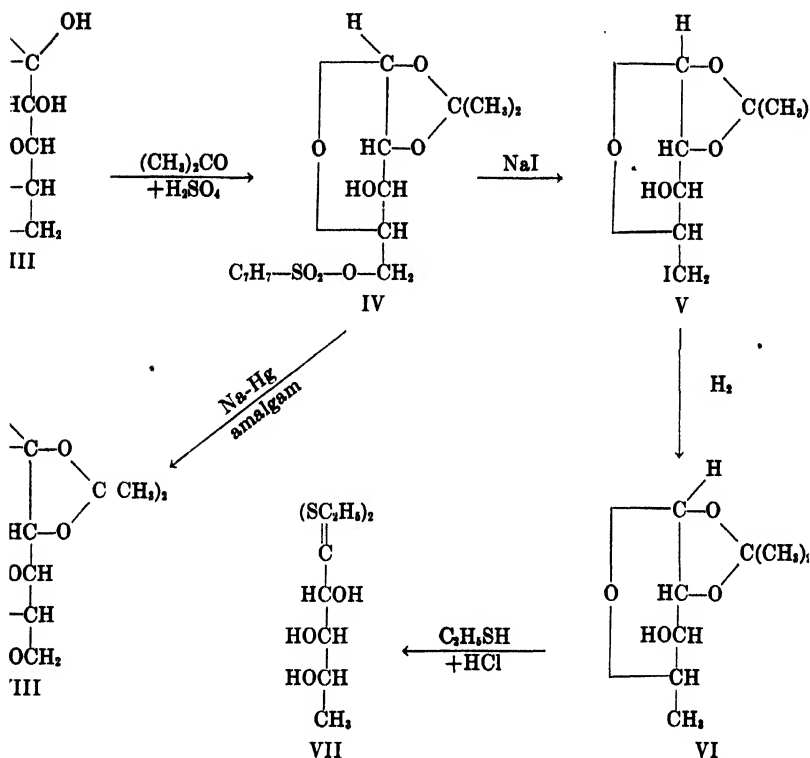


immediately separates, which serves to characterize this sugar derivative.

The condensation of 5-tosyl *l*-arabinose with acetone in the presence of acid and anhydrous copper sulfate results in the formation of crystalline 5-tosyl monoacetone *l*-arabinose (IV). The action of sodium iodide in acetone solution³ on (IV) leads to

³ Oldham, J. W. H., and Rutherford, J. K., *J. Am. Chem. Soc.*, **54**, 366 (1932).

the formation of crystalline 5-iodo monoacetone *l*-arabinose (V). The reduction of (V) with Raney's catalyst in the presence of alkali proceeds smoothly⁴ to yield crystalline monoacetone *l*-arabomethylose (VI). The conversion of (VI) into the easily crystallizable substance, *l*-arabomethylose diethylmercaptal⁵ (VII) is



accomplished through simultaneous hydrolysis and mercaptal formation. The knowledge of the structure of (VII)⁶ carries with it information of the structure of 5-tosyl *l*-arabinose diethylmercaptal.

The second method employed in establishing the position of

⁴ Levene, P. A., and Compton, J., *J. Biol. Chem.*, **111**, 325 (1935).

⁵ Ruff, O., *Ber. chem. Ges.*, **35**, 2362 (1902).

⁶ Swan, D. R., and Evans, W. L., *J. Am. Chem. Soc.*, **57**, 200 (1935).

the tosyl group in 5-tosyl *l*-arabinose diethylmercaptal has been as follows: 5-tosyl *l*-arabinose diethylmercaptal was converted into 5-tosyl methyl-*l*-arabinoside by the procedure of Pacsu.⁷ This substance was then acetylated to yield 5-tosyl diacetyl methyl-*l*-arabinoside and converted to 5-iodo diacetyl methyl-*l*-arabinoside by the method described above. Simultaneous deacetylation and reduction of the iodo compound with Raney's catalyst in the presence of alkali gave crystalline α -methyl-*l*-arabomethyloside identical with that described by Swann and Evans.⁶

The reductive detosylation⁸ of 5-tosyl monoacetone *l*-arabinose gives a new crystalline monoacetone *l*-arabinose (VIII). This substance is characterized by the presence of a furanose ring structure in contrast with the pyranose ring structure ascribed to diacetone *l*-arabinose. Inasmuch as it has never been possible to determine the actual structure of diacetone *l*-arabinose by conventional methods, a careful study of the rate of hydrolysis of this substance with monoacetone *l*-arabofuranoside was made (Table I). The two possible structures for diacetone *l*-arabinose are represented by formulæ (IX) and (X).

Since it has been shown in many cases that the propylidene residue in position <1,2> is much more stable than in any other positions in the sugar molecule, it follows that if (X) is the structure of diacetone *l*-arabinose, then upon hydrolysis (VIII) is first formed and the over-all rate will be conditioned by the rate of hydrolysis of this substance, or that it should be identical with monoacetone *l*-arabofuranoside (VIII). A comparison of the hydrolysis constants of (IX) with (VIII) shows that the latter is hydrolyzed about 4 to 5 times as fast as the former, which excludes the possibility of (X) being the structure of diacetone *l*-arabinose. These hydrolysis data confirm the assumption⁹ that diacetone *l*-arabinose contains the <1,5> pyranose ring structure (IX).

Acetylation of 5-tosyl *l*-arabinose with acetic anhydride in pyridine solution yields sirupy 5-tosyl triacetyl-*l*-arabinose which

⁷ Pacsu, E., *Ber. chem. Ges.*, **58**, 509 (1925).

⁸ Levene, P. A., and Compton, J., *J. Am. Chem. Soc.*, **57**, 2306 (1935).

⁹ Fischer, E., *Ber. chem. Ges.*, **28**, 1166 (1895). Freudenberg, K., and Svanberg, O., *Ber. chem. Ges.*, **55**, 3239 (1922). Svanberg, O., and Bergmann, S., *Chem. Zentr.*, **1**, 1021 (1924).

was converted into sirupy 5-iodo triacetyl-*l*-arabinose by the procedure described above. The transformation of the 5-iodo compound into sirupy 5-nitro triacetyl-*l*-arabinose (b.p. 145–150° at 0.3 mm.) was readily accomplished. Work directed toward the synthesis of nucleosides containing the pentofuranose structure by this method is now in progress.

EXPERIMENTAL

5-Tosyl l-Arabinose Diethylmercaptal—40 gm. of *l*-arabinose diethylmercaptal (m.p. 125–126°) were dissolved in 240 cc. of dry pyridine and 32 gm. (1 mole) of tosyl chloride dissolved in 120 cc. of dry pyridine added at 0°. The mixture was shaken well, allowed to stand at 15° overnight, and poured, with vigorous stirring, into 3 liters of ice water. After standing for a few minutes, a yellow oil separated which soon crystallized, and the product was removed by filtration, washed several times with ice water, and allowed to air-dry on the suction filter. To remove the last traces of pyridine from the product it was necessary to dissolve the crystalline material in 250 cc. of chloroform and wash consecutively with ice-cold 5 per cent sulfuric acid solution, saturated sodium bicarbonate solution, and, finally, ice water. The chloroform extract was dried over anhydrous sodium sulfate, filtered, and diluted to 1 liter with petroleum ether (b.p. 30–40°). After standing a few minutes crystallization began and the solution set to a solid crystalline mass. The product was then removed by filtration and washed several times with a 1:4 mixture of chloroform-petroleum ether. Yield 45 gm. M.p. 65–66°, unchanged by further recrystallization. The specific rotation of the substance in *U. S. P.* chloroform was

$$[\alpha]_D^{25} = \frac{+2.08^\circ \times 100}{2 \times 2.120} = +49.05^\circ$$

The composition of the substance agreed with that of a tosyl pentose diethylmercaptal.

5.122 mg. substance: 8.775 mg. CO₂ and 2.910 mg. H₂O

4.804 " " : 8.140 " BaSO₄

C₁₈H₂₈O₈S₂. Calculated. C 46.78, H 6.38, S 23.41

410.4 Found. " 46.72, " 6.35, " 23.28

The substance is soluble in all the usual organic solvents, insoluble in petroleum ether and water. It is very sensitive to acid and, unless well protected, soon decomposes spontaneously.

5-Tosyl l-Arabinose—40 gm. of 5-tosyl *l*-arabinose diethylmercaptal were dissolved in 120 cc. of acetone containing 40 cc. of water and 80 gm. of washed and dried cadmium carbonate. A solution of 96 gm. of mercuric chloride dissolved in 120 cc. of acetone was then added under good stirring. The mixture was stirred overnight at room temperature, after which the temperature was maintained at 50° for 4 hours. The cooled solution was then filtered, concentrated under diminished pressure at 40° to about 75 cc., and diluted with water to 800 cc. The small amount of unchanged material separating was removed by filtration and the cooled solution treated with hydrogen sulfide to remove the cations present. The filtered solution was then aerated, the acid neutralized with silver carbonate, filtered, treated with hydrogen sulfide, filtered, and concentrated under diminished pressure at 40° to a thick sirup. The product was dried by dissolving in absolute ethyl alcohol and concentrating under diminished pressure. This process was repeated several times and finally the last traces of alcohol were removed by dissolving the product in dry toluene and concentrating to a thick sirup. The substance could not be induced to crystallize and usually contained traces of salts. Yield 18 to 22 gm.

5-Tosyl l-Arabinose Phenylhydrazone—0.5 gm. of sirupy 5-tosyl *l*-arabinose was dissolved in 10 cc. of water and 0.25 gm. (1.5 moles) of phenylhydrazine dissolved in 1 cc. of glacial acetic acid added. The hydrazone immediately crystallized from solution and was removed by filtration. The product was thoroughly washed with water, dried over calcium chloride, and recrystallized from ethyl acetate-petroleum ether (b.p. 30–40°). Yield 0.6 gm. The melting point, after a second recrystallization, was 115–116°, with decomposition. The specific rotation of the substance in dry pyridine was

$$[\alpha]_D^{25} = \frac{+0.16^\circ \times 100}{2 \times 1.268} = +6.31^\circ$$

changing after 24 hours to

$$[\alpha]_D^{25} = \frac{+0.48^\circ \times 100}{2 \times 1.268} = +18.9^\circ$$

The solution upon further standing became too dark for the rotation to be observed. The composition of the substance agreed with that of a tosyl pentose phenylhydrazone.

4.704 mg. substance:	9.500 mg. CO ₂ and 2.330 mg. H ₂ O
6.200 " " :	0.392 cc. N ₂ (25° and 751 mm.)
C ₁₈ H ₂₂ O ₆ N ₂ S. Calculated.	C 54.79, H 5.63, N 7.10
394.2 Found.	" 55.07, " 5.54, " 7.16

The substance is very soluble in all the usual organic solvents, insoluble in petroleum ether and water. It is very unstable and, after standing at room temperature for some time, decomposes readily.

5-Tosyl Monoacetone l-Arabinose—2.0 gm. of sirupy 5-tosyl *l*-arabinose were dissolved in 50 cc. of acetone containing 0.2 per cent of sulfuric acid and 4 gm. of anhydrous copper sulfate. The mixture was shaken at room temperature for 20 hours, filtered, the acid neutralized with calcium hydroxide, and the solution filtered and concentrated under diminished pressure to dryness. The product was then dissolved in a small volume of ethyl acetate, treated with charcoal, filtered, and petroleum ether added until the solution became faintly turbid. After standing in the ice box a short time, the product crystallized in long prismatic clusters. Yield 1.8 gm. M.p. 129–130°, unchanged by further recrystallization. The specific rotation of the substance in U. S. P. chloroform was

$$[\alpha]_D^{25} = \frac{-1.41^\circ \times 100}{2 \times 2.026} = -34.8^\circ$$

The composition of the substance agreed with that of a tosyl monoacetone pentose.

3.912 mg. substance:	7.510 mg. CO ₂ and 2.020 mg. H ₂ O
5.088 " " :	3.412 " BaSO ₄
C ₁₈ H ₂₀ O ₇ S. Calculated.	C 52.32, H 5.88, S 9.31
344.2 Found.	" 52.35, " 5.77, " 9.22

The substance is very soluble in chloroform, ethyl alcohol, ethyl acetate, and benzene; insoluble in petroleum ether and water.

5-Nitro Triacetyl-l-Arabinose—12 gm. of 5-tosyl *l*-arabinose were dissolved in 85 cc. of dry pyridine and 42 cc. of acetic anhydride added at 0°. After standing overnight at room temperature the mixture was poured into 500 cc. of ice water and allowed to stand for 1 hour in the cold. The sirupy product was then extracted with chloroform and the extract freed of pyridine by washing with ice-cold 5 per cent sulfuric acid solution, saturated sodium bicarbonate solution, and finally ice water. After drying over anhydrous sodium sulfate, the chloroform extract was filtered and concentrated under diminished pressure at 40° to a thick sirup which would not crystallize. Yield 14.0 gm.

14.0 gm. of 5-tosyl triacetyl-*l*-arabinose were dissolved in 80 cc. of acetone containing 14 gm. of sodium iodide and heated at 100° in a sealed tube for 1 hour. The tube was then cooled, opened, and the salts removed from the acetone solution by filtration. After thorough washing of the salts, the combined filtrates were concentrated under diminished pressure to a solid mass which was extracted with hot chloroform and the chloroform extract thoroughly washed with water. After drying over anhydrous sodium sulfate, the solution was concentrated under diminished pressure to a thick sirup which distilled only with decomposition under 0.05 mm. Purification by distillation was thus impossible. Yield 13.0 gm.

13.0 gm. of 5-iodo triacetyl-*l*-arabinose were dissolved in 45 cc. of acetonitrile containing 13.0 gm. of silver nitrate. The mixture was then heated at 100° for 4 hours, after which a test portion showed that all the halogen had been removed. The solution was filtered and the silver salts thoroughly washed with acetonitrile. The solution was then concentrated under diminished pressure at 40° to about 10 cc. and diluted with a large volume of chloroform. The chloroform extract was then thoroughly washed with water, dried over anhydrous sodium sulfate, filtered, and concentrated under diminished pressure to a thick sirup which distilled under a high vacuum. B.p. 145–150° at 0.05 mm. Yield 10.0 gm. The specific rotation of the substance in chloroform was

$$[\alpha]_D^{25} = \frac{-0.23^\circ \times 100}{2 \times 2.416} = -4.8^\circ$$

The composition of the substance agreed fairly well with that of a nitro triacetyl-pentose.

4.321 mg. substance: 6.610 mg. CO₂ and 1.850 mg. H₂O

6.005 " " : 0.221 cc. N₂ (28° and 757 mm.)

C₁₁H₁₈O₁₀N. Calculated. C 41.10, H 4.67, N 4.36

321.1 Found. " 41.71, " 4.79, " 4.15

Monoacetone l-Arabinose—1.5 gm. of 5-tosyl monoacetone *l*-arabinose were dissolved in 80 cc. of 80 per cent methyl alcohol and 20 gm. of 4 per cent sodium-mercury amalgam added. The solution was stirred at room temperature for 14 hours and the excess alkali neutralized with a stream of carbon dioxide. The solution was then concentrated to dryness under diminished pressure at 40° and was dried in the usual manner by addition of absolute ethyl alcohol, followed by concentration. The solid material resulting was thoroughly extracted with warm absolute ethyl alcohol, and the extract filtered and concentrated under diminished pressure to a solid mass. This material was then extracted with hot, dry ether until a test portion of the extract no longer gave a residue after evaporation. The combined ether extracts were then filtered and allowed to evaporate on the steam bath. Crystallization began as the concentration proceeded, and, after cooling, the product was removed by filtration. Yield 0.8 gm. M.p. 117–118°. The specific rotation of the substance in water was

$$[\alpha]_D^{25} = \frac{-1.18^\circ \times 100}{2 \times 2.044} = -28.9^\circ$$

The composition of the substance agreed with that of a mono-acetone pentose.

5.016 mg. substance: 9.305 mg. CO₂ and 3.295 mg. H₂O

C₈H₁₄O₆. Calculated. C 50.49, H 7.40

190.1 Found. " 50.58, " 7.35

The substance is soluble in methyl alcohol, ethyl alcohol, and water, very difficultly soluble in ether, and insoluble in petroleum ether. It does not reduce boiling Fehling's solution but, after a short hydrolysis with dilute mineral acids, it gives a strong reduction.

Attempted Condensation of Monoacetone l-Arabinose with Acetone in Presence of Anhydrous Copper Sulfate—2.0 gm. of monoacetone *l*-arabinose (m.p. 117–118°) were dissolved in 400 cc. of acetone and 40 gm. of anhydrous copper sulfate added. The mixture was shaken for 2 days at room temperature, after which the solution was filtered and concentrated to dryness under diminished pressure. The solid material separating was dissolved in a large volume of dry ether and the solution was filtered and concentrated to a small volume on the steam bath. The crystalline material separating was removed by filtration. The recovered product was 1.5 gm. M.p. 116–117°. A small residue which did not dissolve in ether was found to give a strong Fehling's reduction which indicated the presence of free arabinose in the final product.

5-Iodo Monoacetone l-Arabinose—2.0 gm. of 5-tosyl monoacetone *l*-arabinose were dissolved in 20 cc. of acetone containing 2.0 gm. of sodium iodide. The solution was then heated in a sealed tube at 100° for 6 hours, after which the tube was cooled, opened, and the solution filtered. The salts separating were thoroughly washed on the filter and the filtrate concentrated to dryness under diminished pressure at 40°. The solid mass was then thoroughly extracted with hot chloroform, and the extract dried over anhydrous sodium sulfate, filtered, and concentrated to a thick sirup under diminished pressure. The sirup was then taken up in a small volume of ethyl acetate and diluted with petroleum ether (30–40°). After standing for some time the product crystallized in long needles. Yield 1.8 gm. M.p. 66–67°. A second recrystallization of the substance from this solvent did not change the melting point. The specific rotation of the substance in U. S. P. chloroform was

$$[\alpha]_D^{25} = \frac{+0.28^\circ \times 100}{2 \times 2.04} = +6.86^\circ$$

The composition of the substance agreed with that of an iodo monoacetone pentose.

4.404 mg. substance:	5.200 mg. CO ₂	and 1.850 mg. H ₂ O
4.320 " "	: 3.389 " AgI	
C ₈ H ₁₃ O ₄ I.	Calculated.	C 32.00, H 4.37, I 42.30
300.0	Found.	" 32.19, " 4.70, " 42.40

The substance is very soluble in ethyl acetate, chloroform, methyl alcohol, and ethyl alcohol, slightly soluble in petroleum ether and hot water.

Monoacetone l-Arabomethylose—1.5 gm. of 5-iodo monoacetone *l*-arabinose were dissolved in 20 cc. of methyl alcohol and placed in a hydrogen reduction apparatus with a suspension of Raney's nickel catalyst and 5 cc. of 10 per cent sodium hydroxide solution. The apparatus was immediately closed and the solution shaken for 1 hour in an atmosphere of hydrogen under a slight pressure. The theoretical quantity of hydrogen was absorbed in about 30 minutes, whereupon no further absorption occurred. The solution was then filtered, and the excess alkali neutralized with a stream of carbon dioxide and concentrated to dryness under diminished pressure at 40°. After thorough drying, the solid material was thoroughly extracted with hot dry ether, and the ether extract dried over anhydrous sodium sulfate, filtered, and concentrated to a small volume under diminished pressure. Crystallization immediately began and after one recrystallization from ether the product gave a constant melting point of 83–84°, with sintering at 80°. Yield 0.5 gm. The specific rotation of the substance in water was

$$[\alpha]_D^{25} = \frac{-0.28^\circ \times 100}{1 \times 2.006} = -13.9^\circ$$

The composition of the substance agreed with that of a monoacetone pentomethylose.

5.216 mg. substance: 10.510 mg. CO₂ and 3.695 mg. H₂O

C₈H₁₄O₄. Calculated. C 55.17, H 8.04

174.0 Found. " 54.94, " 7.92

The substance is soluble in all the usual organic solvents with the exception of petroleum ether and fairly soluble in cold water. It does not reduce boiling Fehling's solution, but, after hydrolysis with dilute mineral acids, it gives a strong reduction.

Conversion of Monoacetone l-Arabomethylose into l-Arabomethylose Diethylmercaptan—200 mg. of monoacetone *l*-arabomethylose were dissolved in 0.5 cc. of concentrated hydrochloric acid (*d* 1.18) and allowed to stand 5 minutes at room temperature. The solution was then cooled to 0° and 0.4 cc. of ethylmercaptan

added with vigorous shaking. After about 5 minutes a few small pieces of ice were added, whereupon crystallization began. The mixture was then diluted with 3 to 4 cc. of water, filtered, and washed with ice water. The dried product after one recrystallization from hot water gave a melting point of 108–109°, unchanged by further recrystallizations. Yield 180 mg. The specific rotation of the substance in dry pyridine was

$$[\alpha]_D^{25} = \frac{+0.23^\circ \times 100}{1 \times 1.936} = +11.9^\circ$$

The composition of the substance agreed with that of a pentomethylose diethylmercaptal.

4.660 mg. substance: 7.690 mg. CO₂ and 3.500 mg. H₂O

4.382 " " : 8.485 " BaSO₄

C₉H₂₀O₅S₂. Calculated. C 44.96, H 8.45, S 26.64

240.2 Found. " 45.00, " 8.40, " 26.60

Conversion of 5-Tosyl l-Arabinose Diethylmercaptal into α-Methyl-l-Arabomethylloside—10.0 gm. of 5-tosyl *l*-arabinose diethylmercaptal were dissolved in 250 cc. of methyl alcohol and 20 gm. of mercuric chloride dissolved in 25 cc. of hot methyl alcohol added to the boiling solution. The mixture was allowed to reflux for 10 minutes, cooled, and filtered. Dry hydrogen sulfide was then passed into the cold solution until precipitation was complete, the solution filtered, aerated, the acid neutralized with silver carbonate, and finally filtered again. The clear filtrate was then concentrated under diminished pressure to a thick sirup which was dissolved in chloroform, dried over calcium chloride, filtered, and concentrated again to a thick sirup. The product could not be crystallized but the properties of the substance indicated it to be a tosyl methylglycoside. Yield 7.5 gm.

4.0 gm. of the sirupy 5-tosyl methyl-*l*-arabinoside were dissolved in 30 cc. of dry pyridine and 14 cc. of acetic anhydride added at 0°. After standing overnight at room temperature, the mixture was poured into 400 cc. of ice water and the oily material separating was extracted with chloroform. The chloroform extract was freed of pyridine in the usual manner and, after finally being washed with water, was dried over anhydrous sodium sulfate, filtered, and concentrated under diminished pressure to a thick sirup which could not be crystallized. Yield 5.0 gm.

5.0 gm. of sirupy 5-tosyl-2,3-diacetyl methyl-*l*-arabinoside were dissolved in 50 cc. of acetone containing 5 gm. of sodium iodide. The mixture was heated in a sealed tube at 100° for 1.5 hours, after which the tube was cooled, opened, and the sodium salts removed by filtration. The filtrate was then concentrated under diminished pressure to a solid mass which was thoroughly extracted with chloroform, filtered, and washed with water. After drying over calcium chloride, the extract was filtered and concentrated under diminished pressure to a thick sirup which did not crystallize. Yield 4.5 gm.

4.5 gm. of 5-iodo 2,3-diacetyl methyl-*l*-arabinoside were dissolved in 80 cc. of methyl alcohol and 20 cc. of 10 per cent sodium hydroxide solution added with a suspension of Raney's catalyst in methyl alcohol. Reduction was carried out with shaking at room temperature and under slightly more than 1 atmosphere of pressure. The theoretical volume of hydrogen was absorbed after about 15 minutes and the reaction was stopped after 30 minutes. The solution was then filtered, the excess alkali neutralized with a stream of carbon dioxide, and the solution concentrated to dryness under diminished pressure at 40°. The dry solid was then thoroughly extracted with hot chloroform, and the chloroform extract dried over calcium chloride, filtered, and concentrated under diminished pressure to a thick sirup which distilled completely under a high vacuum. B.p. 95–100° at 0.3 mm. The distillate crystallized completely after standing a short time and was recrystallized from dry ether. The melting point was 88–89°, unchanged by further recrystallization. Yield 0.6 gm. The specific rotation of the substance in U. S. P. chloroform was

$$\alpha_D^{25} = \frac{-2.60^\circ \times 100}{2 \times 1.00} = -130.0^\circ$$

The composition of the substance agreed with that of a methylpentomethylsido.

4.582 mg. substance: 8.179 mg. CO₂ and 3.350 mg. H₂O

2.987 " " : 11.95 cc. 0.01 N Na₂S₂O₄

C₆H₁₂O₄. Calculated. C 48.54, H 8.17, OCH₃ 20.93

148.1 Found. " 48.67, " 8.18, " 20.67

The substance is non-reducing toward Fehling's solution, but after hydrolysis with dilute mineral acid gives a strong reduction.

*Comparison of Rates of Hydrolysis of Diacetone l-Arabinose*¹⁰ with Monoacetone *l*-Arabinose—0.6008 gm. of diacetone *l*-arabinose was dissolved in exactly 20.0 cc. of 0.01 N hydrochloric acid solution and heated in a glass-stoppered flask at 54°. At the intervals of time specified in Table I, portions of the solution were removed, immediately cooled to room temperature, and the specific rotation observed.

TABLE I

Rate of Hydrolysis of Diacetone l-Arabinose and Monoacetone l-Arabinose with 0.01 N HCl at 54°

$$k = (2.303/t) \log_{10} (\alpha_i - \alpha_f) / (\alpha_i - \alpha_f).$$

Time	$[\alpha]_D^{25}$		$k \times 10^{-3}$	
	Diacetone <i>l</i> -arabinose	Monoacetone <i>l</i> -arabinose	Diacetone <i>l</i> -arabinose	Monoacetone <i>l</i> -arabinose
<i>min.</i>	<i>degrees</i>	<i>degrees</i>		
0	+6.5	-27.7		
30	+7.3	-2.6		
90	+13.4	+24.9		
150		+46.4		7.3
210	+23.9	+59.9	1.6	7.0
300		+71.1		7.3
390	+35.9		1.6	
570	+45.6		1.7	
α^*	+68.8	+83.4		

* Final equilibrium values are equivalent to $[\alpha]_D^{25} = +105.8^\circ$ calculated on the basis of 0.1956 gm. of free *l*-arabinose being liberated in each case.

A sample of this solution after 30 minutes hydrolysis gave a strong Fehling's reduction, showing that the propylidene residue in position <1,2> had been hydrolyzed.

In a similar manner the rate of hydrolysis of 0.2482 gm. of monoacetone *l*-arabinose dissolved in 10.0 cc. of 0.01 N hydrochloric acid solution was observed. The results obtained are given in Table I.

¹⁰ We wish to thank Dr. R. S. Tipson of this laboratory for the sample of very pure diacetone *l*-arabinose (m.p. 42-43°) used in this determination.

THE HIGHER SATURATED FATTY ACIDS OF BUTTER FAT*

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Butter fat has been found to be a very complex fat, containing in its glyceride linkages a large number of fatty acids. Since some of these acids are present in very small amounts, they have in the past been frequently overlooked. This is especially true of the fatty acids of higher molecular weight than stearic acid.

Hilditch and Jones (1), Hilditch and Sleightholme (2), and others have reported the presence in butter fat of small amounts (0.5 to 1.0 per cent) of saturated fatty acids of higher molecular weight than stearic acid. Since these amounts have been calculated from the mean molecular weights, it has been assumed that this material consisted largely of the next higher acid than stearic acid; *i.e.*, arachidic acid. Bosworth and Brown (3), after a somewhat extended study of the acids derived from their highest boiling methyl ester fraction (230–285° at 15 mm.) concluded that lignoceric acid and possibly behenic and cerotic acids were present in butter fat.

Bosworth and Sisson (4) confirmed the presence of behenic acid.

EXPERIMENTAL

A preliminary study of the high molecular weight saturated fatty acids was made on certain fractions, from the series prepared by Bosworth and Sisson (4). The fractions studied in this work were not included in the report of Bosworth and Sisson. These fractions were saponified, the soaps extracted with ether to remove the non-saponifiable material, and the saturated acids isolated by means of the lead soap-ether separation. The data are found in Table I.

* The greater part of this paper has been taken from a dissertation presented by George E. Helz in partial fulfilment of the requirement for the degree of Doctor of Philosophy in the Graduate School of the Ohio State University.

An inspection of Table I shows that maximum yields were obtained for Fractions 4 and 7. The mean molecular weights of these fractions indicate the presence of behenic and tetracosanoic (lignoceric) acids, respectively. Fractions 7 and above contained acids that had mean molecular weights higher than that of tetracosanoic acid, indicating the presence of a hexacosanoic acid or a mixture of acids of higher molecular weight. The low mean molecular weight of the saturated acids in the residue was shown to be due to acids of low molecular weight, indicative of the presence of anhydrides, decomposition products, or unesterified glycerides in the residue. Since these ester fractions consisted of

TABLE I
Yield of Methyl Esters and Molecular Weights of Saturated Fatty Acids

Fraction No.	Boiling range of methyl esters at 5 mm. pressure	Weight of esters	Mean mol. wt. of saturated acids
	°C.	gm.	
1	220-225	11.4	327
2	225-230	9.4	315
3	230-235	10.0	329
4	235-240	16.0	342
5	240-245	10.0	342
6	245-250	8.3	345
7	250-255	17.0	377
8	255-260	10.5	387
9	260-265	8.8	388
10	265-270	4.8	388
11	270-275	1.1	388
Residue	Above 275	94.0	265

mixtures of esters of unsaturated acids as well as saturated acids, the total weight of saturated acids obtainable was too small for a more complete study. In order to obtain more material for the study of the saturated acids as well as the unsaturated acids, arrangements were made to work on a larger batch of butter.

Study of High Molecular Weight Material from Methyl Esters from 360 Pounds of Butter—The methyl esters of the fatty acids from 360 pounds of butter were carefully fractionated at 15 mm. pressure. The undistilled residue consisting of 5680 gm. of material not distilling below 215° at 15 mm. pressure was used for the work herein reported. This material was saponified and the soaps

extracted with ether until the ether washings were negative to the Liebermann-Burchard reaction for cholesterol.

The acids were then recovered, esterified, and the esters carefully distilled at 5 ± 0.1 mm. pressure. Fourteen fractions and a residue were obtained. Fractions 1 to 8 were redistilled three times and Fractions 9 to 14 inclusive, four times. Pyrolysis started at 245° in each case, so the distillations were stopped at that temperature. Since it is known that the methyl esters of the higher molecular weight saturated acids will distil over unchanged at a temperature higher than 245° at 5 mm., this pyrolysis observed must have been due to methyl esters of unsaturated acids of high molecular weight which were known to be present.

TABLE II
Data Obtained from Ester Fractions and Saturated Acids

Fraction No. *	B.p. at 5 mm. pressure	Total weight of esters	Weight of saturated acids obtained	Mean mol. wt. of saturated acids
	$^\circ\text{C.}$	<i>gm.</i>	<i>gm.</i>	
5	195-200	348	128	284
6	200-205	248	100	294
7	205-210	193	77	312
8	210-215	59	23	313
9	215-220	115	45	322
10	220-225	175	83	335
11	225-230	118	63	341
12	230-235	82	48	359
13	235-240	38	25	369
14	240-245	26	17	367
Residue	Above 245	450		

Fractions 1 to 4 inclusive were found to have mean molecular weights indicative of the presence of methyl stearate and methyl esters of lower acids, and were therefore eliminated from further analysis. Fractions 5 to 14 inclusive were saponified, the acids recovered, and the acids subjected to a lead soap-ether separation. The data are given in Table II.

An inspection of Table II shows that saturated acids from C_{18} (mol. wt. 284.4) to C_{24} (mol. wt. 368.5) are to be found in these mixtures. The maximum yield obtained for Fraction 10 corresponds to the presence of a fairly large amount of behenic acid (mol. wt. 340). It is interesting to note that the maximum yield

corresponding to the presence of methyl behenate in this instance was obtained at a temperature about 15° lower than that at which the maximum yield was obtained by Bosworth and Sisson (see Table I). This may be explained by the fact that in the present experiment the distillation apparatus was more carefully regulated and also that the present batch of esters had been freed of cholesterol and other non-saponifiable matter.

Analysis of Residue Remaining above 245° at 5 Mm.—Approximately 450 gm. of esters remained as a residue not distilling over below 245° . This material was saponified in alcohol with strong potassium hydroxide. The acids were then recovered.

The acids were dissolved in 2500 cc. of boiling acetone. A small amount of white flaky material remained insoluble. This in-

TABLE III
Data Secured from Saturated Acids in Residue after Crystallization from Acetone

	Mol. wt.	Melting point
		$^{\circ}\text{C.}$
Recovered saturated acids.....	391.9	79
1st crystallization (crystals).....	390.3	79
1st " (mother liquor).....	436.5	
2nd " (crystals).....	392.3	80.5
2nd " (mother liquor).....	386.4	
9th "	396.4	80.5

soluble material was found to consist of unsaponified methyl esters. The whole batch of material was therefore resaponified and the acids recovered. The recovered acids were found to be totally soluble in boiling acetone. The data obtained on the crystallized acids are given in Table III.

The crystals from acetone consisted of coralloid clumps of short blunt needles, microscopic in size. The acid was next recrystallized from alcohol. The crystals from alcohol were similar in appearance to those obtained from acetone except that they were smaller in size. The melting point of the crystals from alcohol was also 80.5° .

From the constancy of the melting point and the mean molecular weight the material was considered to be practically pure cerotic acid.

Preparation of the Amide—The amide was prepared by boiling 5 gm. of the acid with 15 cc. of thionyl chloride for 12 hours. The resulting solution was poured into an excess of strong aqueous ammonia (sp.gr. 0.90). The amide was filtered off and washed repeatedly with cold water and finally with hot water to remove any ammonium soaps. The material was then extracted with ether for 40 hours in a Soxhlet apparatus to remove any unchanged acids. The amide melted at 105–107°.

Preparation of the Methyl Ester—15 gm. of the acid and 100 cc. of methyl alcohol containing 4 per cent dry HCl were boiled under a reflux condenser for 24 hours. A large amount of water was then added and the ester taken up in ether. This was washed in a separatory funnel until free of HCl. The ether was then removed by warming the flask on a water bath, the suction from a water pump being used to remove as much of the solvent as possible.

TABLE IV
Data Obtained from Saponification of Methyl Esters

	Mean mol. wt. of esters	Mean mol. wt. of acids (calculated)
Distillate.....	410.9	396.9
Residue.....	412.2	398.2

The esters were then transferred to a 50 cc. distilling flask and distilled at carefully regulated pressures.

The boiling point at 15 ± 0.1 mm. pressure was 286°. The boiling point at 5 ± 0.1 mm. pressure was 261°. About 10 gm. of the material were distilled over, the remainder being allowed to remain in the distilling flask as a residue. Samples from the distillate and from the residue were saponified to determine the mean molecular weight. The results are given in Table IV.

Both of the values obtained for mean molecular weights (Table IV) correspond closely to that for cerotic acid (396.4), indicating that the material was fairly pure.

Small amounts of esters from both the distilled portion and the residue were crystallized from boiling methyl alcohol. The melting point of the methyl ester was found to be 62° in each case.

The remaining esters were saponified and the acids recovered. The acids obtained from the distilled portion of the methyl esters

after recrystallization from acetone were found to consist of microscopic nacreous crystalline plates. The acids obtained from the residual portion were similar in appearance but also contained some granular material. No doubt the original crystallized hexacosanoic acid had contained a small amount of impurity which remained in the residual portion.

SUMMARY

1. Further information has been presented regarding the saturated fatty acids of higher molecular weight than stearic acid found in butter fat.

2. Hexacosanoic (cerotic) acid has been isolated from butter fat. This acid crystallizes as nacreous crystalline plates from acetone and has a melting point of 80.5° . The methyl ester distills at 286° at 15 ± 0.1 mm. pressure and at 261° at 5 ± 0.1 mm. pressure. The melting point of the methyl ester is 62° . The amide melts at $105-107^{\circ}$.

3. There is definite evidence that hexacosanoic (cerotic) acid is the higher molecular weight saturated fatty acid found in the butter investigated.

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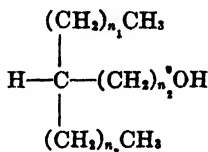
ROTATORY DISPERSION OF CONFIGURATIONALLY RELATED ALIPHATIC CARBINOLS

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It was the aim of the present investigation to establish the direction of rotation of configurationally related carbinols of the general formula



having $n_2 = 0$ with those having $n_2 \geq 1$. The problem cannot be solved by the methods of classical organic chemistry. A satisfactory solution was reached on the basis of the change in sign of rotation observed in the members of the series of carbinols homologous with respect to n_1 , having $n_2 = 1$. In this series the first and the higher members differ in the direction of their rotation (*cf.* Table I). Similar events have been observed previously in other homologous series such as β -disubstituted propionic acids,¹ sulfo acids,² and alkyl azides having $n_1 = 0$ and $n_2 = 1$.³ In these series, the corresponding partial rotations in all members are of the same sign. However, the factors behind the observed change in sign of rotation in this series of primary alcohols are of a different nature.

In the carbinols the difference in the sign of rotation in the visible region of the first and of the higher members is due to the

¹ Levene, P. A., Rothen, A., and Marker, R. E., *J. Chem. Physic.*, **16**, 662 (1933).

² Levene, P. A., and Rothen, A., *J. Chem. Physic.*, **2**, 681 (1934).

³ Levene, P. A., and Rothen, A., unpublished results.

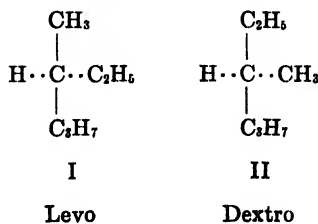
fact that the corresponding partial rotations of the hydroxyl group of the first member are of opposite sign from those of the higher members. Thus, in the carbinols having $n_2 = 1$, the substitution of the CH_3 group by a C_2H_5 (in position (1)) brings about a change of sign of the partial rotation of the hydroxyl group in the radicle

TABLE I
*Configurationally Related Secondary and Primary Carbinols. $[M]_D^{25}$
Maximum*

CH ₃	CH ₃
H··C··OH	H··C··CH ₂ OH
C ₃ H ₇	C ₃ H ₇
+12.0°	-6.8°
C	A
<hr/>	
C ₂ H ₅	C ₂ H ₅
H··C··OH	H··C··CH ₂ OH
C ₃ H ₇	C ₃ H ₇
+2.0°	+3.8°
D	B



CH_2OH . The effect on rotation is similar to the case of two optically active normal aliphatic hydrocarbons (I) and (II). If hydrocarbon (I) is levorotatory then (II) is dextrorotatory. The difference in the dissymmetry of the two hydrocarbons is the result of the difference in spatial arrangements of the substituents, the order



being clockwise in one and counter-clockwise in the other.⁴ In the two alcohols (A) and (B) the situation is analogous; in one of them the groups are arranged clockwise, and in the other, counter-clockwise (see Table I). Indeed, the configurations of the carbinols can be correlated to those of the hydrocarbons by methods of classical organic chemistry so that there is sufficient reason to believe that in the alcohols having $n_2 = 1$ the direction of rotation is determined by the clockwise or counter-clockwise arrangement of the groups.

With this knowledge it is possible to find the direction of rotation of the corresponding secondary carbinols (C) and (D). In these, the direction of rotation in the visible region is likewise determined by the partial rotation of the hydroxyl groups. The arrangement of the groups in order of their volume is identical in both; hence, both rotate in the same direction.

The arrangement of the groups in (C) is opposite to that in (A); the arrangement in (D) is similar to that in (B). It is therefore logical to assume that the direction of rotation is identical in (B) and (D) and not in (A) and (C). Thus, in the case of (A) and (C) the change of $n_2 = 0$ to $n_2 = 1$ brings about a change in sign of rotation. In the case of (B) and (D) and of the higher homologues with respect to n_1 , the change of $n_2 = 0$ to $n_2 = 1$ does not affect the sign of the direction of rotation.

Partial Rotation of Hydroxyl Group—It was stated above that the direction of rotation of the alcohols is determined by the partial rotation of the hydroxyl. It is important to emphasize at this place first, that the nearest to the visible absorption band of the hydroxyl group is not perceptibly anisotropic; second, that the partial rotation of the hydroxyl group contains at least two rotatory components. When the configurations of the dextro secondary carbinols of the methyl series were correlated to that of the dextro-octanol-4⁵ (the first member of the propyl series) it was found that the latter compound exhibited a levorotation in ether solution.

⁴ The abbreviated expression "clockwise or counter-clockwise" will be used in the following sense. If a molecule is viewed with the largest group toward the observer, the three other groups are arranged in clockwise or counter-clockwise order according to diminishing volume. This is in agreement with the convention adopted by Boys.

⁵ Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **83**, 579 (1929).

The immediate inference was that the course of dispersion of this carbinol was anomalous either in the homogeneous state or in ether. The results of our present study (see Table II) show that the dispersion is anomalous in the homogeneous state and normal in ether. From our previous work on rotatory dispersion of homologous series, it is well established that configurationally related members with the same sign of rotation exhibit the same type of dispersion; *i.e.*, analogous absorption bands function similarly. The important deduction follows that *the dispersion curves of all secondary carbinols are anomalous, and the partial rotation of the first active band is of opposite sign to the observed rotation.* This conclusion could not be drawn from the dispersions of the members of the methyl series, since in this series the first contribution is very small for wave-lengths greater than λ 2500.

However, another series of facts is in favor of the view that secondary carbinols have an anomalous rotatory dispersion. Kenyon and Barnes⁶ prepared a series of ethers from dextro-nonanol-3 (from methyl up to nonyl ether). All the ethers were dextrorotatory with the exception of the methyl ether which was levorotatory. The dextro ethers exhibited typical anomalous dispersion, whereas the dispersion of the methyl ether was normal, the two corresponding partial contributions being of the same sign as in the higher ethers. It is warranted to assume that what is valid for the ethers also holds for the corresponding carbinols, inasmuch as the substitution of an alkyl group for a hydrogen atom does not introduce into the molecule a group having an absorption band in the longer wave-length region.

The concept of the existence of two rotatory components of opposite sign in the hydroxyl group of the secondary carbinols, even though the first be of very low value, may be very significant, inasmuch as it can serve to bring into harmony the conclusions of Kuhn⁷ on the absolute configurations of secondary carbinols with those of Boys.⁸

Assuming that the first rotatory component of the hydroxyl is of

⁶ Kenyon, J., and Barnes, T. W., *J. Chem. Soc.*, **125**, 1395 (1924).

⁷ Kuhn, W., *Z. physik. Chem., Abt. B.*, **31**, 23 (1935).

⁸ Boys, S. F., *Proc. Roy. Soc. London, Series A*, **144**, 675 (1934).

TABLE II

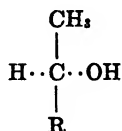
Rotatory Dispersion of Configurationally Related Secondary Carbinols

Dextro-butanol-2* in homogeneous state. $d_4^{25} = 0.8032$ (in vacuo); $n_D^{25} = 1.3953$; visible region, $l = 20$ cm.; ultraviolet region, $l = 10$ cm.; $[M]_{\max}^{25} = \frac{3.3124}{\lambda^2 - 0.0245}$			Dextro-octanol-4 in homogeneous state. $d_4^{25} = 0.8159$ (in vacuo); $n_D^{25} = 1.4228$; visible region, $l = 20$ cm.; ultraviolet region, $l = 10$ cm.; $[M]_{\max}^{25} = \frac{0.2633}{\lambda^2 - 0.005}$			Levo-octanol-4 in ether. Concentration = 0.8286 M; $l = 100$ cm.; $[M]_{\max}^{25} = -\frac{0.1448}{\lambda^2 - 0.041}$	
λ	α^{25}	$[M]_{\max}^{25}$	λ	α^{25}	$[M]_{\max}^{25}$	α^{25}	$[M]_{\max}^{25}$
	degrees	degrees		degrees	degrees	degrees	degrees
5780.1	17.874	10.697	5875.6	0.977	0.779	-0.404(?)	-0.487(?)
5460.7	20.232	12.108	5780.1	1.002	0.799	-0.410	-0.495
4358.3	33.46	20.02 ₆	5460.7	1.126	0.898	-0.467	-0.563
4046.6	39.76	23.79	4358.3	1.785	1.423	-0.805	-0.971
3885	21.90	26.21	4046.6	2.08	1.66	-0.955	-1.15
3685	24.90	29.80	3940	1.12	1.79		
3375	30.90	36.98	3370	1.52	2.42		
3258	33.90	40.57	3200	1.72	2.74		
3068	39.90	47.76	3030	1.92	3.06		
2985	42.90	51.35	2870	2.12	3.38		
2843	48.90	58.53	2780	2.32	3.70		
2786	51.90	62.12	2350	0.30†	4.8		
2749	53.90	64.51					
2650	6.00†	71.8					
2610	6.30†	75.4					
2520	7.00†	83.8					

* The $[M]_{\max}^{25}$ value is that given by Pickard and Kenyon (Pickard, R. H., and Kenyon, J. K., *J. Chem. Soc.*, **103**, 1924 (1913)). It should be mentioned incidentally that the data given for the dispersion of this compound in Pickard and Kenyon's article and which have since appeared (Lowry, T. M., *Bur. Standards, Bull. 118* (1932)) are erroneous. These data are in complete disagreement with the ratio $\alpha_{4358.3}:\alpha_{5460.7} = 1.662$ given by Lowry (Lowry, T. M., *J. Chem. Soc.*, **105**, 94 (1913)). Our own value $\alpha_{4358.3}:\alpha_{5460.7} = 1.6538$ is appreciably different from Lowry's. We are inclined to hold our own figures as more correct inasmuch as the value 1.6538 is nearer the value 1.651 which was found consistently by Lowry for all the higher members of the same series.

† 1 cm.

opposite direction from that of the carbinol in the visible region, it follows that the carbinol



should be dextrorotatory on the basis of the model of Kuhn as well as on the basis of the assumption of Boys.

Analysis of Rotatory Dispersions—It is known that the absorption bands of carbinols are located in the very distant ultraviolet region, the weak first band being in the neighborhood of λ 1800. Hence, the analysis of the rotatory dispersion curves of such substances proved difficult and it was only by comparison of the dispersion of a number of homologues that information concerning the relative values of the different rotatory components could be obtained.

The data regarding the dispersions of the tested carbinols are summarized in Tables II to V.

Secondary Carbinols with $n_2 = 0$, $n_1 = 0$ or 2 , $n_3 = 1$ or 2 —Only one carbinol of the methyl series was tested. As can be seen from Table II, the dispersion curve can be well expressed by a single term Drude formula from λ 5780 to λ 2600, deviations less than 1 per cent occurring for the smallest wave-lengths. The dispersion constant was found to correspond to λ 1560. From measurements in the visible region, Lowry, Pickard, and Kenyon⁹ found approximately the same value for a number of carbinols of the methyl as well as of the ethyl series.

The rotatory dispersion of the dextro-octanol-4 was found, as mentioned above, anomalous in homogeneous state and normal in ether solution, the rotations being of opposite sign. Both curves could be expressed for the wave-length interval studied by a single Drude term formula having $\lambda_0^2 = 0.005$ and 0.041 , respectively.

Primary Carbinols with $n_2 = 1$, $n_1 = 0$ or 1 , $n_3 = 1, 2$, or 3 —In spite of the fact that for all the visible and near ultraviolet regions the dispersion curves can be expressed by a single term Drude

⁹ Lowry, T. M., Pickard, R. H., and Kenyon, J. K., *J. Chem. Soc.*, **105**, 94 (1914).

formula whose dispersion constant corresponds to about λ 1800, it should be concluded from the strong curvature of the curve $1/\alpha = f(\lambda^2)$ for the distant ultraviolet region that the first

TABLE III

Rotatory Dispersion of Configurationally Related Carbinols with $n_D = 1$

λ	Levo-2-methylbutanol-1 in homogeneous state.* $\alpha_D^{25} = 0.8158$ (in vacuo); $n_D^{25} = 1.4084$; visible region, $l = 20$ cm.; ultraviolet region, $l = 10$ cm.			Levo-2-methylpentanol-1 in heptane. $n_D^{25} = 1.4185$; concentration = 1.951 M; visible region, $l = 20$ cm.; ultraviolet region, $l = 5$ cm.; $[\alpha]_{\max}^{25} = -\frac{2.157}{\lambda^2 - 0.028}$	
	α^{25}	$[\alpha]_{\max}^{25}$	$[\alpha]_{\max}^{25} = -\frac{1.6262}{\lambda^2 - 0.0345}$	α^{25}	$[\alpha]_{\max}^{25}$
	degrees	degrees		degrees	degrees
5875.6	-9.186	-5.234	-5.234	-0.892	-6.80
5780.1	-9.526	-5.428	-5.428	-0.924	-7.04
5460.7	-10.823	-6.167	-6.167	-1.053	-8.03
4358.3	-18.38	-10.47	-10.46	-1.755	-13.38
4046.6	-22.08	-12.58	-12.58	-2.09	-15.93
3780	-13.10	-14.93	-15.00		
3580	-15.10	-17.20	-17.36		
3415	-17.10	-19.49	-19.81		
3285	-19.10	-21.77	-22.15		
3175	-21.10	-24.04	-24.53		
3085	-23.10	-26.32	-26.80		
3060				-1.07	-32.6
3003	-25.10	-28.60	-29.20		
2935	-27.10	-30.88	-31.49		
2864	-29.10	-33.16	-34.22		
2820				-1.32	-40.2
2750	-33.10	-37.72	-39.55		
2675	-3.60†	-41.0	-43.8		
2380	-5.45†	-62.1	-73.6		

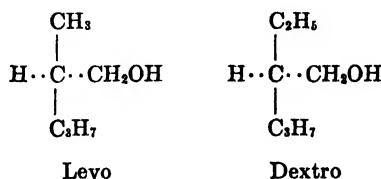
* The molecular rotations are about 5 per cent higher in heptane than in the homogeneous state. Lowry, Pickard, and Kenyon (Lowry, T. M., Pickard, R. H., and Kenyon, J. K., *J. Chem. Soc.*, 105, 94 (1914)) gave the ratio $\alpha_{4358.3}:\alpha_{4460.7} = 1.700$, in good agreement with our own data 1.698.

† 1 cm.

absorption band contributes very little, if at all, to the observed rotation (see Tables III and IV). There is no doubt that the two main contributions are located in the shorter wave-length region

and are of opposite sign; hence the high value of the dispersion constant when a single term is used to represent the dispersion.

In the case of methylpropyl- and in that of ethylpropylethanol, the corresponding anisotropies induced in the bands of the OH group are undoubtedly of opposite sign.



Primary Carbinols with $n_2 = 2$ or 3, $n_1 = 0$ or 1, $n_3 = 1$ or 2—From the above discussion of the rotatory events in the carbinols with

TABLE IV

Rotatory Dispersion of Configurationally Related Primary Carbinols with $n_2 = 1$ (Homogeneous State)

λ	Dextro-2-ethylpentanol-1. $\alpha_D^{25} = 0.8280$ (in vacuo); $n_D^{25} = 1.4250$; visible region, $l = 20$ cm.; ultraviolet region, $l = 10$ cm.; $[M]_{\text{max.}}^{25} = \infty \frac{1.2130}{\lambda^2 - 0.036}$		Dextro-2-ethylhexanol-1. $\alpha_D^{25} = 0.8292_6$ (in vacuo); $n_D^{25} = 1.4292$; visible region, $l = 40$ cm.; ultraviolet region, $l = 10$ cm.; $[M]_{\text{max.}}^{25} = \infty \frac{2.4180}{\lambda^2 - 0.033}$	
	α^{25}	$[M]_{\text{max.}}^{25}$	α^{25}	$[M]_{\text{max.}}^{25}$
	degrees	degrees	degrees	degrees
5875.6	1.394	3.942	5.085	7.757
5780.1	1.440	4.072	5.268	8.036
5460.7	1.640	4.637	5.986	9.131
4358.3	2.79	7.89	10.12	15.44
4046.6	3.38	9.56	12.14	18.52
3790			3.60	22.0
3595			4.10	25.0
3450			4.60	28.1
3330			5.10	31.1
3220			5.60	34.2
3140	3.45	19.5		
3125			6.10	37.2
3060	3.70	20.9		
2970			7.10	43.3
2900	4.45	25.2		
2750			1.85 (2 cm.)	56.4

TABLE V
Rotatory Dispersion of Configurationally Related Primary Carbinols with $n_3 = 2$ or 3

λ	Dextro-3-methylpentanol-1* in homogeneous state. $d_4^{25} = 0.8156$ (in vacuo); $n_D^{25} = 1.4178$; visible region, $l = 20$ cm.; ultraviolet region, $l = 10$ cm.; $[M]_{\max}^{25} = \frac{2.9761}{\lambda^2 - 0.0203}$	Levo-3-methylhexanol-1 in heptane. $n_D^{25} = 1.4230$; concentration = 0.6041 M; $l = 100$ cm.; $[M]_{\max}^{25} = \frac{0.6820}{\lambda^2 - 0.0237}$; equation valid to $\infty 12400$	Dextro-4-methylhexanol-1 in homogeneous state. $d_4^{25} = 0.8222$ (in vacuo); $n_D^{25} = 1.4232$; $l = 10$ cm.; $[M]_{\max}^{25} = \frac{3.830}{\lambda^2 - 0.0242}$	Levo-3-ethylheptanol-1 in homogeneous state. $d_4^{25} = 0.8331$ (in vacuo); $n_D^{25} = 1.4371$; visible region, $l = 20$ cm.; ultraviolet region, $l = 5$ cm.; $[M]_{\max}^{25} = -\frac{1.55}{\lambda^2 - 0.012}$ †		
	α^{25} degrees	$[M]_{\max}^{25}$ degrees	α^{25} degrees	$[M]_{\max}^{25}$ degrees	α^{25} degrees	$[M]_{\max}^{25}$ degrees
5892.6	5.989	9.182	-1.106	-2.100	-1.656	-4.694
5875.6	6.184	9.481	-1.156	-2.195	-1.722	-4.881
5780.1	6.990	10.72	-1.308	-2.483	-1.945	-5.51
5460.7	11.45	17.55	-2.16	-4.10	-3.16	-8.971
4358.3	13.53	20.74			-3.71	-10.53
4046.6	7.90	24.2				
3780	8.90	27.3				
3595	9.90	30.3				
3445						
3420	10.90	33.4				
3310						
3250	11.90	36.5			-1.50	-17.0
3190	13.90	42.6				
2993					-1.90	-21.5
2800						
2840	1.63 (1 cm.)	5.0				

* Rotatory measurements were carried out in heptane solution also. The constant of dispersion was found to be the same but the molecular rotations were 5 per cent higher. Two samples of 3-methylpentanol-1 prepared from a different material were tested. A rather large discrepancy was found in the dispersion of both samples. The sample which was considered as less pure had a constant of dispersion appreciably lower, $\lambda_0^2 = 0.0155$, instead of the adopted value, $\lambda_0^2 = 0.0203$.

$n_2 = 1$, one understands why 3-methylpentanol-1 is of opposite sign from 3-methylhexanol-1 but rotates in the same direction as 4-methylhexanol-1, when configurationally related. One should expect all carbinols of this group to exhibit the same type of dispersion. As seen from Table V, their dispersion can be expressed by a single Drude term formula. Low rotation unfortunately restricted the dispersion studies to a limited wave-length interval. The constant of dispersion is equal to or smaller than that of the corresponding secondary carbinols.

The study of the dispersion of the secondary carbinol ($n_2 = 0$) with $n_1 = 1$ brought out convincing evidence (as mentioned above) that the dispersion of all secondary carbinols of the same general

TABLE VI
Changes in Direction of Individual Rotatory Components Produced by Progressive Increase in n_2

	$n_2 = 0$	$n_2 = 1$	$n_2 > 1$	
			Lower member with respect to n_2	Higher member with respect to n_2
$n_1 = 0$ { 1st component.....	-	--	-	+
2nd "	++	+	++	--
$n_1 = 1$ { 1st "	-	++	-	+
2nd "	++	-	++	--

type is anomalous. In a similar way, the very low value of the dispersion constant of ethylamylpropanol (3-ethyloctanol-1) (see last column, Table V) is in favor of the view that all carbinols with $n_2 = 2$ exhibit an anomalous dispersion.

We recently stated¹⁰ that all lower members of the series of carbinols with $n_2 > 1$ are of opposite sign to those with $n_2 = 1$, owing to the fact that in the former the first partial contribution has a lower numerical value than the second contribution. We should add that this is also valid for the higher members in spite of their being of opposite sign, the change of direction of rotation in this case being due to the change in molecular dissymmetry.

Changes in Directions of Individual Rotatory Components Pro-

¹⁰ Levene, P. A., and Rothen, A., *J. Org. Chem.*, **1**, 76 (1936).

duced by the Progressive Increase in the Value of n_2 —On the basis of the analysis of the rotatory dispersion of the carbinols having $n_2 = 1, 2$, or 3 , it is now possible to differentiate between the directions of rotation of each individual rotatory component in the members of the above series. They are given in Table VI for the carbinols correlated to the dextrorotatory secondary alcohols.

Thus in the case of the carbinols, the changes in the direction of rotation of individual contributions are conditioned by the changes in the molecular dissymmetry. It will be shown later that the rotatory events are totally analogous for the corresponding amines.

THE DETERMINATION OF CYSTINE IN URINE

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At various times methods have been devised for the determination of cystine. Aside from the direct isolation of cystine, all the methods proposed for cystine entail the reduction of cystine to cysteine and determination of the latter. Most of the proposed methods are dependent on the reactive $-SH$ group of the cysteine and accordingly give positive results with reducible disulfides other than cystine.

The Sullivan method (1-3), on the other hand, requires that the three groups of cysteine be unsubstituted and in the naturally occurring order $-SH, -NH_2, -COOH$. The reaction covering cystine is negative with many other disulfides and even with isocystine, homocystine, dithioethylamine (cystine amine), and glutathione, all of which react like cystine in other cystine methods. A positive reaction is naturally given by complexes which readily yield cystine as, for example, cystine dimethyl ester.¹ In quantitative work with proteins, Sullivan (4) and Sullivan and Hess (5,6) showed that most protein hydrolysates require the addition of strong sodium hydroxide before the addition of the final reducing agent. With this modification the Sullivan method has been applied to the quantitative estimation of cystine in the hydrochloric acid hydrolysates of various purified foodstuffs, and of wool, hair, nails, etc. The findings have been checked by the Okuda (7,8) iodometric method (which is satisfactory in most hydrochloric acid hydrolysates). Both methods gave results of the same order of magnitude.

¹ The reason for the stronger reaction with the ester and the consideration of the so called positive reaction with cystinyl peptides will be given in subsequent publications.

Certain modifications of the Sullivan procedure have been made but in no case have we found such modifications or variations as satisfactory as the original quantitative Sullivan method (4). A consideration of the criticism and of the so called improvement in procedure is not pertinent to the present paper which deals with the application of the Sullivan cystine procedure to urine.

The urine offers difficulties not only for the determination of cystine but for the determination of many other ingredients. Early Sullivan, Hess, and Chase (9) found that urine contained material which inhibits more or less the determination of added cystine by the Sullivan method but at the same time found that much greater error occurred in various other methods, such as the Folin-Looney (10), Folin-Marenzi (11), and the Okuda methods. It was found that the inhibiting substances could be eradicated to a high degree by treatment with norit or better carboraffin (12). In the early work the amount of cystine found in normal urines was small. On the other hand, it was found that hydrolyzing the decolorized urine gave a marked increase in cystine as determined colorimetrically. Little attention, however, was paid to the estimation of cystine in urine until the question was brought forcibly to our attention by the work of Brand (13, 14) on cystinurics.

Brand, Cahill, and Block (14) using a slightly modified Sullivan method found that substances in urine somewhat inhibited the color development. They found that homocystine and ascorbic acid lessened the color given by cystine. More recently, without giving any details, Lewis, Brown, and White (15) declare that homocystine depressed the color formation in the Sullivan method but failed to influence the development of color in the Lugg (16) modification of the Sullivan procedure.

The findings of both Brand and of Lewis as to homocystine are difficult to understand, for, as will be shown later, homocystine has absolutely no inhibitory action on the estimation of cystine. On the other hand, as mentioned by Sullivan in various publications, numerous substances will interfere not only with the Sullivan reaction but also, and to a greater degree, with other methods used by some as cystine methods,—the Folin-Marenzi and the Okuda methods. These inhibitory materials, broadly speaking, are highly reducing and highly oxidizing compounds which are rarely if ever present in biological material in sufficient amounts

to interfere and highly buffering complexes such as an excessive amount of certain amino acids. In no hydrolysates of protein, however, has any amount of mixed amino acids been found interfering with the colorimetric cystine determination if the procedure given by Sullivan (4) and by Sullivan and Hess (5, 6) is followed. The consideration of such highly reducing, highly oxidizing agents, and large amounts of amino acids is quite aside from the question of estimating cystine in urine, the procedure for which will be detailed presently.

Homocystine—The proof that homocystine has no inhibiting action on cystine estimation by the Sullivan procedure may be seen from the following experiments.

Cystine and homocystine were dissolved separately in 0.1 N HCl so that each 5 cc. of solution contained 1 mg., respectively. Then equal parts of these solutions were mixed so that each 5 cc. contained 0.5 mg. of cystine and 0.5 mg. of homocystine. To 5 cc. of the mixture were added 2 cc. of 5 per cent aqueous NaCN with thorough mixing. At the end of 10 minutes standing 1 cc. of 0.5 per cent 1,2-naphthoquinone-4-sodium sulfonate was added with shaking for 10 seconds, then 5 cc. of 10 per cent anhydrous Na_2SO_3 in 0.5 N NaOH. After mixing, by shaking, the solution was allowed to stand for 30 minutes. Then 1 cc. of a 2 per cent solution of sodium hyposulfite ($\text{Na}_2\text{S}_2\text{O}_4$) in 0.5 N NaOH was added. Compared with a cystine solution containing 0.5 mg. in 5 cc. similarly treated the readings were 20 and 20.1. Repeated as before but with 2 cc. of the naphthoquinone the readings were 20 and 19.8.

Cystine and Homocystine Added to Urine—Cystine and homocystine were added to urine in the proportion used by Brand *et al.* (14). Thus 16.25 mg. of cystine and 5.5 mg. of homocystine were added to 50 cc. of urine. Neither substance went completely into solution, so any work with the urines would be questionable. Accordingly, to the 50 cc. of urine plus cystine and homocystine (Solution A) 1 cc. of concentrated HCl was added and the mixture was gently heated for 5 minutes and then made to 50 cc. with water. 4 cc. were used for colorimetric estimation with urine containing the same amount of cystine (Solution B) and similarly treated as the standard. The cystine found colorimetrically in the Sullivan procedure (with aqueous cyanide and 1 cc. of the

naphthoquinone 0.5 per cent aqueous solution) was the same in Solutions A and B but only 80 per cent of the color given by 1.3 mg. of cystine in 4 cc. of 0.1 N HCl. Practically the same result was obtained with the Lugg modification. The retardation of color was not due to homocystine but to other urinary conditions which call for greater alkalinity and more naphthoquinone.

Effect of Homocystine on Determination of Cystine with Use of Greater Alkalinity and More Naphthoquinone—To 20 cc. of urine were added 6.25 mg. of cystine and 2.2 mg. of homocystine. The mixture was warmed until solution was complete and made to 20 cc., Solution C. 5 cc. of Solution C were diluted to 10 cc. with 0.1 N HCl. To this 10 cc. and to 10 cc. of original urine were added 4 cc. of 5 per cent NaCN in N NaOH and the solutions were centrifuged, stirred, and recentrifuged. When 10 minutes had elapsed, 7 cc. of each solution were used for colorimetric work. To each 7 cc. were added 2 cc. of 1 per cent naphthoquinone with shaking for 10 seconds, then 5 cc. of Na_2SO_3 in 0.5 N NaOH, and after 30 minutes standing at room temperature (24°) 2 cc. of 5 N NaOH followed by 1 cc. of 2 per cent sodium hyposulfite ($\text{Na}_2\text{S}_2\text{O}_4$) in 0.5 N NaOH. The urine (without addition) matched against a 100 parts per million standard (0.5 mg. of cystine in 5 cc.) similarly treated showed 111 parts per million. With allowance for the cystine normally present the recovery of added cystine was 95 per cent despite the fact that a little of the cystine was occluded by the alkaline urinary precipitate. It can be said safely that homocystine has little if any inhibiting action on the Sullivan reaction.

Ascorbic Acid—Brand *et al.* (14) and Andrews and Andrews (17) call attention to the inhibiting action of ascorbic acid on the Sullivan cystine reaction. This phenomenon we had noted when using 1 mg. of ascorbic acid to 1 mg. of cystine per each 5 cc., but found that the inhibition could be easily offset by using a 5 per cent solution of sodium cyanide in N NaOH, mixing, and waiting 10 minutes, then adding 2 cc. of 1 per cent naphthoquinone and continuing as in the regular cystine procedure. In this modification with a cystine standard containing 0.5 mg. per 5 cc. set at 20 mm. the solution containing 0.5 mg. of cystine and 0.5 mg. of ascorbic acid per 5 cc. read 20.2. The alkaline cyanide converts the ascorbic acid into a cyanhydrin complex of little reducing

capacity. It may be said that the inhibiting action of urinary constituents on the Sullivan cystine reaction cannot be due to ascorbic acid, since rarely, if ever, is there enough ascorbic acid in the urine, a statement we shall give proof of in a subsequent publication dealing with the determination of ascorbic acid in general.

What the interfering material in urine is we do not know but with the following procedure cystine in urine and cystine added to urine can be estimated with a high degree of exactness by the Sullivan method.

Sullivan Cystine Method Applied to Urine—To 10 cc. of urine add 4 cc. of 5 per cent NaCN in N NaOH, stir, centrifuge 3 minutes, restir, and recentrifuge. When 10 minutes have elapsed from the time of adding the cyanide, take 7 cc. of the supernatant liquid for colorimetric determination.

To 7 cc. add 2 cc. of 1 per cent 1,2-naphthoquinone-4-sodium sulfonate, shake for 10 seconds, add 5 cc. of 10 per cent anhydrous Na_2SO_3 in 0.5 N NaOH, and wait 30 minutes. Then add 2 cc. of 5 N NaOH, shake, and add 1 cc. of 2 per cent $\text{Na}_2\text{S}_2\text{O}_4$ in 0.5 N NaOH. Match against 5 cc. of an appropriate cystine standard plus 2 cc. of alkaline sodium cyanide and treated as in the above colorimetric work. The results by this procedure for five different 24 hour urines collected over 10 cc. of chloroform are given in Table I for the urine as voided and after various treatments.

In Column 3 the cystine findings for the untreated urines are given. The urine after standing for 24 hours shows an increase of 37 per cent. In Column 5 are given the cystine findings after gently boiling 20 cc. of urine with 2 cc. of concentrated HCl for 20 minutes on a hot-plate, neutralizing, and making to 20 cc. with water. This short heating with slight acidity gives a large increase over the untreated urine. In the hydrolysis of these urines, 20 cc. of urine were heated with 20 cc. of concentrated HCl without a reflux until the total volume was about 10 cc., then with the reflux attached the solution was further heated for 5 hours; then it was carefully neutralized to pH 3.5 and made to 20 cc. with 0.1 N HCl. A much increased cystine content is found. In Column 7 the data obtained by the Okuda method are given. This method has to have special treatment when applied to urine. The original urine is difficult to reduce by Zn and HCl but can be readily reduced if norit is added as recommended by Okuda (8). The pro-

cedure is as follows: To 10 cc. of urine plus 1 cc. of concentrated HCl add 30 mg. of carboraffin (a carbon superior to norit for this purpose) and a small amount of Zn powder; reduce 1 hour at room temperature, filter, and wash, and continue as given by Okuda (7). This method, in unhydrolyzed solutions, is not a cystine method but is a measure of total —SH compounds and certain other complexes of high reducing capacity in an acid medium.

Recovery of Cystine Added to Urine—Sample A: To 25 cc. of a normal urine which invariably is higher in cystine than other normal urines were added 5 mg. of cystine. The cystine did not go readily into solution, so the urine was acidified by addition of 0.5

TABLE I
Cystine Content of Urine; Unheated, Heated, Hydrolyzed

Subject No.	Volume	Cystine in total 24 hr. urine				
		Original urine	After 24 hrs.	After acidifying and heating	After hydrolysis	Okuda method on original urine
(1)	(2)	(3)	(4)	(5)	(6)	(7)
	cc.	mg.	mg.	mg.	mg.	mg.
1	850	94.35	112.54	129.34	147.13	159.21
2	1120	41.22	60.48	65.59	69.81	81.46
3	1680	68.54	99.79	104.43	94.08	106.90
4	1795	66.42	92.98	99.44	97.38	125.83
5	2310	75.77	109.03	121.51	148.14	145.50
Average...	1551	69.26	94.96	104.06	111.31	123.79

cc. of N HCl and was then heated for 5 minutes, and on cooling made to 25 cc. with water. Sample B: 25 cc. of the same urine, without addition of cystine, similarly acidified and heated. Sample C: 25 cc. of the same urine plus cystine, acidified but not heated. Sample D: 25 cc. of the same urine untreated.

Then 10 cc. of each sample were treated with 4 cc. of 5 per cent NaCN in N NaOH and the mixture centrifuged. Without washing of the sediment 7 cc. were used for colorimetric work. With the use of 100 parts per million standard for the urine alone and a 200 parts per million for the urine plus cystine the findings were as follows: Sample D, 0.3845 mg. of cystine in 5 cc.; Sample C, 1.111 mg. of cystine in 5 cc.; Sample B, 0.397 mg.; Sample A, 1.212 mg.

The recovery of the added cystine was 72.7 per cent for the unheated urine, 81.5 per cent for the heated.

This experiment was repeated but in addition the centrifuged sediment was washed in various ways as follows: Sample E with 10.0 cc. of 0.1 N HCl followed by 4.0 cc. of 5 per cent NaCN in N NaOH, centrifuged again, and decanted into the original supernatant liquid. Sample F with 5.0 cc. of the HCl and 2.0 cc. of the cyanide; Sample G, the sediment that came on adding 4.0 cc. of the 5 per cent alkaline cyanide to the urine and centrifuging 3 minutes was thoroughly stirred and recentrifuged; Sample H, washing the sediment with 7.0 cc. of 0.5 N NaOH. When 7.0 cc. of the supernatant liquid and appropriate standards were used, the added cystine and total cystine accounted for were: Sample E 97 and 98 per cent, Sample F 93.3 and 95 per cent, Sample G 97 and 98 per cent, Sample H 86 and 91 per cent.

The fact that the precipitate that forms on adding cyanide to a urine may occlude cystine has been ignored by other workers in this field. With some normal urines the loss by occlusion is negligible, but with urines containing cystine and considerable calcium phosphate the loss may be great.

Lewis, Brown, and White (15) report that they were unable to detect cystine in combined form or cystine reacting more slowly than the usual form of cystine. These findings are not in agreement with the work of Brand *et al.* (13) nor with data in the present paper. Their cystinuric urine, however, is different and possibly their findings are correct, while ours are correct for the normal urines. It is conceivable that in their case all the substances which yield cystine have already done so without further treatment. In such a case Lewis, Brown, and White would find no increase on standing or on hydrolyzing. However, when applied to our normal urines the procedure of Lewis, Brown, and White was found highly unsatisfactory. Thus, with the use of alkali (pH 12.5) to precipitate the phosphate and with the Lugg modification of the Sullivan method we find liberation of cystine from complexes and more or less loss of cystine in the sediment. Experiments may be cited to indicate the validity of these statements. Thus, in the use of the Lugg modification, when 60 mg. of glutathione (GSH) were added to 100 cc. of a synthetic urine (see Okuda (8)) also containing 20 mg. of cystine, 27.5 per cent

of the theoretical cysteine (estimated as cystine) was obtained from the glutathione. In the procedure given in this present paper the same synthetic urine containing cystine and glutathione yielded less than 1.0 per cent of the theoretical cysteine of the glutathione to colorimetric estimation (calculated as cystine). When an old sample of oxidized glutathione was analyzed in a similar way the Lugg modification showed a relatively large liberation of cystine from the glutathione, while the procedure used in this paper showed only a small liberation.

Glutathione added to normal urine containing little cystine yields only a small amount of its cysteine to colorimetric estimation. With a urine which normally shows about 100 parts per million of cystine, added glutathione increased the colorimetric findings from 5 to 10 per cent in the procedure given in this paper. Secondly, a urine which by the Sullivan procedure (with use of alkaline cyanide and 2.0 cc. of a 1.0 per cent naphthoquinone solution) showed 104 parts per million of cystine indicated 112 parts per million after the alkaline treatment and neutralization with N HCl as used by Lewis.

In the Lugg modification, the shades of color in the standard and the urine are different, the one a brownish red, the other with a high yellow tint. Matching different shades is always questionable. Without prejudice as to the value of the findings, and matching intensity of colors, this particular urine in the Lugg modification gave 109 parts per million before alkali treatment and 124 parts per million when put through the Lewis, Brown, and White procedure, despite the fact that cystine is occluded in the precipitate, as will be presently shown. In our hands the Lewis procedure gave increases over the non-alkalinized urine greater than making the urine 1.7 per cent acid and heating for 10 minutes, a phenomenon which might be interpreted as indicating the decomposition, in whole or in part, of a cystine complex as labile as glutathione. If such decomposition occurred in the case of Lewis, Brown, and White, they would find little increase on aging or hydrolyzing, since their cystinuric urine is already saturated with cystine and minimizes any added cystine from complexes.

In the Lewis, Brown, and White procedure applied to our urines there is more or less occlusion of cystine in the precipitate. Thus

a urine containing 1.0 mg. of added cystine to each 5.0 cc., treated with alkali, and centrifuged without washing the sediment gave in the urinary procedure described in this paper a recovery of added cystine of only 60 per cent, while washing the precipitate with 6.0 cc. of 0.17 N NaOH and adding 1 cc. of N HCl to the supernatant liquid and adding this to the original centrifugate gave a return of 98 per cent of the added cystine. The same urine treated with alkaline cyanide and with the sediment washed with 10.0 cc. of 0.1 N HCl and 4.0 cc. of alkaline cyanide gave a recovery of 95 per cent when put through the colorimetric procedure given in this paper.

Further, in the Lewis procedure with colorimetric determination by means of the Lugg modification of the Sullivan reaction this urine as voided indicated no cystine present. This finding is erroneous, since the precipitation with cuprous chloride (18) and getting rid of the copper by H_2S showed 74.9 parts per million by the Sullivan method and 74.3 by the Okuda, while the Sullivan method modified for urine and applied directly to the urine showed 79.2 parts per million.

The Lugg modification of the Sullivan cystine procedure is essentially Procedure 1 of Sullivan's (1) original paper and considered by him too lacking in delicacy to catch small amounts of cystine. Several times, in fact, we have compared the Lugg modification with the original alkali-hyposulfite method given by Sullivan and found them in practical agreement. Both the Lugg modification and the alkali hyposulfite method show less than 50 per cent as much color as the sodium sulfite procedure. With 50 parts per million of cystine, the Lugg modification is absolutely negative, whereas the original Sullivan sodium sulfite procedure as given in Paper IV (4) of his sulfur studies gives a good red color at this concentration. With normal urines which show cystine by other procedures, the Sullivan direct method and the Sullivan and Okuda procedures on the cuprous chloride precipitate, freed from copper by H_2S , the Lugg modification is negative. The Sullivan and the Okuda methods on the Cu_2Cl_2 precipitate gave 94.5 per cent of the value found in the original urine by the procedure given in this paper. Our conclusion is that the Lugg modification is unsuitable for normal urines and that even with urines containing 1 mg. per each 5 cc. the Lewis-Lugg procedure is

very questionable because of the loss of cystine and the poor color shade.

Aside from the possibility that in the cystinuric case of Lewis, Brown, and White all cystine has been liberated, there is another explanation of Lewis' failure to find increase of cystine on allowing the urine to stand. His urines did not stand more than several hours. We have followed urines from the time of voiding for 22 hours. An example of our findings is given in Table II.

Estimation of Cystine in Urine by Precipitating with Cuprous Chloride Followed by Colorimetric Estimation—Rossouw and Wilken-Jorden (18) precipitate cystine by means of cuprous chloride and after getting rid of copper by KCNS estimate the cystine by a slight modification of the Sullivan method. In our hands, elimi-

TABLE II

Change in Cystine Content of Normal Urines on Standing

The urine samples were collected at 11 a.m.

Subject No.	Volume	Cystine found				Ratio of initial to final
		11 a. m.	1 p. m.	3 p. m.	After 22 hrs.	
	cc.	mg.	mg.	mg.	mg.	
1	160	15.23	15.92	17.52	19.63	100:129
2	52	2.81	2.84	2.84	3.51	100:125
3	210	8.90	8.98	9.77	11.17	100:126

nating the copper by means of KCNS gave only 92 per cent of the value found when the copper was eliminated by hydrogen sulfide. With hydrogen sulfide instead of KCNS, on an average of eight separate urines the cuprous chloride procedure gave 92 per cent of the value given by the present procedure in the case of fresh urines and 85 per cent of the value given by the urine standing overnight.²

There is a question in our minds as to whether the cuprous chloride applied to urine precipitates the cystine quantitatively. The cuprous chloride will not precipitate reduced or oxidized glutathione or certain other simple cystinyl complexes added to urine, either synthetic or natural. It does precipitate glycylcystine. This peptide does not give the Sullivan cystine reaction, though

² With a number of compounds added to urine we have found evidence of a slow tying up to form simple complexes.

positive in other cystine methods. The difference between the procedure used in this paper and the results on the cuprous chloride precipitate could be taken to indicate the presence of a cystine complex not precipitated by cuprous chloride and giving reactive cystine in an alkaline medium.

These results indicate that the Rossouw-Wilken-Jorden procedure gives slightly lower than actual values for free cystine, while the Sullivan-Hess procedure gives both free cystine and whatever cystine is readily liberated from simple complexes. Since the cuprous chloride precipitates homocystine, other so called cystine methods are unsatisfactory for the determination of cystine, even in the cuprous chloride treatment, if homocystine is present. In a few normal urines tested no evidence was obtained for the presence of homocystine.

SUMMARY

1. Because of the buffering power of the urine, the presence of reducing material, and of substances combining with 1,2-naphthoquinone-4-sodium sulfonate, the original Sullivan cystine procedure, when applied to urine, must be modified by using an alkaline cyanide for reduction, with washing of the sediment occurring on addition of alkaline cyanide, and by the use of more naphthoquinone for full color development.

2. Normal urines contain a small amount of free cystine.

3. Normal urines contain cystine complexes which liberate cystine on standing, and also on treatment with acid and alkali.

4. Hydrolyzing urine gives a considerable increase in the cystine content determined colorimetrically.

5. Evidence is given that homocystine does not interfere with the determination of cystine in the Sullivan method and that the interference by ascorbic acid can be easily prevented.

6. The Lugg-Sullivan procedure is too lacking in delicacy to be applied to normal urine. Even with added cystine the color of the urine is different from that given by the same amount of cystine in the standard.

7. Unless the sediment is washed, urine containing appreciable amounts of calcium phosphate may by occlusion give erroneous values if treated with alkali.

8. Cystine added to urine is recovered to a high degree of accuracy.

9. The Rossouw-Wilken-Jorden procedure gives cystine values slightly too low.

10. The procedure given in this paper shows both free cystine and cystine complexes yielding cystine in an alkaline medium.

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AN EXAMINATION OF THE SULLIVAN COLORIMETRIC TEST FOR GUANIDINE

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Under the above title, Braun and Rees (1) tested the Sullivan (2) guanidine reaction on thirty-six guanidine complexes and concluded that the reaction given by Sullivan as highly specific for unsubstituted guanidine was given with certain substituted guanidines, *p*-aminophenylguanidine sulfate, α -naphthylguanidine hydrochloride, *ar*-tetrahydro- α -naphthylguanidine hydrochloride, *o*-tolylguanidine hydrochloride, *p*-tolylbiguanide hydrochloride. All of these are synthetic compounds and normally would not occur in biological fluids. They are of interest, however, in testing the theory that the Sullivan guanidine reaction is given only by unsubstituted guanidine or compounds that yield guanidine readily.

Since close derivatives of the unsubstituted guanidine did not give the reaction, it seemed possible (a) that the compounds of Braun and Rees were split up to yield free guanidine in the procedure employed in the guanidine test or (b) that the reaction was not that of guanidine. On receipt of the five compounds in question from Dr. Braun it was found that they did in fact give a red color in the final step which would prevent determination of guanidine. However, the reaction was not a guanidine reaction for no one of the compounds behaved like guanidine in the different steps and all of them could be readily made negative. In the testing, the respective compounds were dissolved in water to give the equivalent of 1 mg. of free unsubstituted guanidine per cc.

On adding the naphthoquinone to *p*-aminophenylguanidine sulfate a heavy reddish precipitate formed. Guanidine at this stage gives a light orange solution. Accordingly, the complex formed by addition of 2 cc. of 1,2-naphthoquinone-4-sodium sulfonate (1 per

cent aqueous solution) to 2 cc. of *p*-aminophenylguanidine sulfate was centrifuged and decanted. The sediment, stirred, was recentrifuged, and decanted. This total resulting solution was 2.4 cc. 2 cc. were put through the guanidine test with a negative result.

Of the preparations, α -naphthylguanidine hydrochloride, *ar*-tetrahydro- α -naphthylguanidine hydrochloride, *o*-tolylguanidine hydrochloride, and *p*-tolylbiguanide hydrochloride, α -naphthylguanidine hydrochloride and *ar*-tetrahydro- α -naphthylguanidine hydrochloride gave a slight red at the stage of addition of HCl, while guanidine gave a strong red and precipitate, and *o*-tolylguanidine hydrochloride and *p*-tolylbiguanide hydrochloride were practically negative. On addition of HNO₃, all four gave a red coloration as mentioned by Braun and Rees. However, all of these guanidine preparations are negative in the guanidine test if a small amount of hydroxylamine is added before the acidification, as follows: To 1 cc. of each solution, containing the equivalent of 1 mg. of unsubstituted guanidine, add 1 cc. of a freshly made solution of 1,2-naphthoquinone-4-sodium sulfonate, shake, and add 0.3 cc. of *N* NaOH and heat 1 to 2 minutes in a water bath, cool, and add 0.2 cc. of a 20 per cent aqueous solution of hydroxylamine HCl, shake, and add 0.5 cc. of concentrated HCl and 0.5 cc. of concentrated HNO₃, shake for 1 minute, and add 3 cc. of 95 per cent alcohol. α -Naphthylguanidine hydrochloride, *ar*-tetrahydro- α -naphthylguanidine hydrochloride, *o*-tolylguanidine hydrochloride, and *p*-tolylbiguanide hydrochloride were yellow; that is, negative. Guanidine gave a strong red color. *p*-Aminophenylguanidine sulfate gave a red precipitate and a yellow solution on centrifuging, while guanidine centrifuged yields a strong red solution. The guanidine solution plus the hydroxylamine matched the guanidine solution put through the regular procedure, *i.e.* with 0.2 cc. of 25 per cent urea solution instead of hydroxylamine. The color of the product obtained in the regular procedure, however, fades less rapidly.

The hydroxylamine was employed specifically to show that the compounds of Braun and Rees can readily be made negative and thus readily differentiated from unsubstituted guanidine. Its value in guanidine work in general is problematical. There are probably other compounds, especially those which give color with nitric acid, which unless given special treatment will inter-

fere, but such compounds so far have not been met with in biological solutions.

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STUDIES ON THE CHEMISTRY OF BLOOD COAGULATION

III. THE CHEMICAL CONSTITUENTS OF BLOOD PLATELETS AND THEIR RÔLE IN BLOOD CLOTTING, WITH REMARKS ON THE ACTIVATION OF CLOTTING BY LIPIDS*

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The blood platelets, or thrombocytes, are the third formed element of mammal blood. Since their discovery by Hayem in 1877 (1) and by Bizzozero in 1882 (2), considerable work has been done concerning their origin, morphology, and function (3, 4). While most of these questions still are controversial, it has been well established that the platelets play an important part in the extravascular and intravascular clotting of blood (4). They are considered to be the sole source, or, with the leucocytes possibly playing a minor rôle, the main source of the substance which activates prothrombin to thrombin and which has variously been called thromboplastin, thrombokinase, thrombozyme, cytozyme, etc.

The difficulty of obtaining these fragile corpuscles in sufficient amount has prevented any thorough chemical investigation. Apart from microscopical findings and work with emulsions of platelets (see *e.g.* (5)), the importance for blood clotting which has been assigned to these cells in various theories has been corroborated by very little experimental evidence.

From a chemical study of blood platelets not only a deeper understanding of the mechanism of blood clotting can be expected, but also an important contribution to our knowledge concerning the formation of thromboses where the so called platelet thrombi

* Study of the mechanism of thrombosis and embolism supported by the Carnegie Corporation of New York.

play an important rôle (6). The only chemical data on platelets to be found in the literature are contained in a paper by Abderhalden and Deetjen (7) on the proteases of platelets and in a short note by Haurowitz and Sládek (8) according to whom horse thrombocytes contain 71 per cent of proteins, 12 per cent of lipids, 1.7 per cent of cholesterol (determined by colorimetry), and 5.5 per cent of ash.

In the present paper a study is presented of the various chemical constituents, primarily the lipids, of platelets from horse blood, together with experiments on the importance of these fractions for the clotting of blood. The main difficulty encountered in this investigation was the extreme scarcity of material, which made it necessary to control almost every step in the isolation and purification of the various fractions by conducting model experiments with mixtures of similar substances from other sources.

A number of phosphatides of plant and bacterial origin also were examined as to their effect on blood clotting in order to compare their activity with that of the platelet preparations.

EXPERIMENTAL

Composition of Blood Platelets

Preparation of Material—The starting material consisted of the upper layer of sedimented blood cells obtained from large amounts of oxalated horse blood. The operations had to be repeated several times until enough material was available. We describe here one experiment only. The cells were suspended in a chilled solution containing 0.8 per cent of sodium chloride and 0.6 per cent of sodium citrate. A solution of similar composition which is far superior to the ones containing higher citrate concentrations has been used by Hamburger (9) in his fundamental work on leucocytes. The cell suspension is placed in high glass cylinders and kept in the cold for a few hours. The suspension separates into a leucocyte layer on the bottom, a thin grayish white layer above, and a turbid supernatant liquid. Both the supernatant liquid and the narrow upper layer are drawn off separately and submitted to cell counts. The leucocyte-platelet quotient is usually found between 1:60 and 1:200. These suspensions are centrifuged in the angle centrifuge at 3000 R.P.M. for 20 minutes and the white precipitates are washed twice with the ice-cold citrate solu-

tion. The sediments from all suspensions, the cell counts of which are within the range just mentioned, are united, resuspended in citrate solution, and submitted to sedimentation. (All the operations are carried out in the cold.) The bottom layer consisting of leucocytes and coagulated platelets is discarded; the milky supernatant liquid contains one leucocyte in 350 platelets. By repeating the operations described more platelet material is obtained from the bottom layer of the first sedimentation. The suspensions with a leucocyte-platelet quotient of about 1:350 are again united. The centrifugation, washing, suspension, and sedimentation are repeated five to eight times, until the counts show the presence of one leucocyte in 3000 to 4000 platelets. The actual contamination of the material with leucocytes is even smaller, as the platelets have the tendency to clump together and thereby escape the counting. The final suspensions are centrifuged, washed once with physiological saline, and suspended in a mixture of equal amounts of absolute alcohol and peroxide-free ether. The preparation of the material lasts about 72 hours.

In this manner two batches of blood platelets were secured, which had a calculated total dry weight of 472 mg. and 493 mg. respectively. There is no doubt that only a small fraction of the platelets present in our starting material could be obtained at this degree of purity.

Isolation of Lipids—The platelets were extracted with alcohol-ether in the dark at room temperature for 21 days. (All the operations were carried out, as far as possible, in N_2 atmosphere.) They were then filtered off and extracted with chloroform for the same period of time. Two individual batches of platelets were worked up, as mentioned above, and the distribution of the various fractions is presented in Table I. Although the fractions isolated in both series of experiments were practically identical, it will be seen that the amounts in which the substances were isolated from the material varied considerably. This certainly is partly due to a difference in the manner of isolation.

In Experiment I (Table I) the alcohol-ether extract was concentrated *in vacuo*, diluted with water, and repeatedly extracted with ether. An extremely tenacious emulsion formed, which could only partly be broken. By a comparatively large volume of ether 8.5 mg. only of lipid material were extracted. A larger

fraction (25.8 mg.) was given off when the aqueous layer was acidified with dilute H_2SO_4 . But even then the aqueous emulsion obviously retained part of the lipids. As the main difference between the results of Experiments I and II lies in the phosphatide fraction, it appears probable that at least part of this fraction entered the aqueous phase in a complex water-soluble form. The combined *lipids* weighed 34.3 mg. and formed a light brown soft mass.

In Experiment II (Table I) the alcohol-ether extract was concentrated to dryness *in vacuo* and the residue treated with ether. An ether-insoluble fraction, 7.6 mg. of a white powder, was elimi-

TABLE I
Composition of Blood Platelets

Fraction No.	Fractions	Platelets			
		Experiment I		Experiment II	
		mg	per cent dry material	mg.	per cent dry material
1	Lipids (soluble in alcohol-ether)	34.3	7.3	82.6	16.8
2	" (" " chloroform)	7.1	1.5	1.2	0.2
3	Phosphatides (insoluble in acetone)	17.5	3.7	59.1	12.0
4	Lecithin	10.4	2.2	34.9	7.1
5	Cephalin	5.5	1.2	23.3	4.7
6	Acetone-soluble fraction	16.6	3.5	22.6	4.6
7	Cholesterol	8.7	1.8	13.9	2.8
8	Defatted platelets	431.1	91.2	408.7	83.0

nated by centrifugation. This fraction was inactive in blood clotting. It proved to be the emulsifying agent which had made extraction so difficult in Experiment I. The ethereal solution, when washed with water, formed almost no emulsions. From it 83.6 mg. of *lipids* were obtained.

The *chloroform-soluble lipids* were yellow oils which were entirely inactive in blood clotting and were not further examined.

The *defatted platelets* formed an almost white, extremely light powder. The question of the activity of aqueous extracts of this material will be discussed later in this paper.

Separation of Lipids—In order to save space, only Experiment II will be described. The lipids (83.6 mg.) were dissolved in 1

cc. of ether, and 2 cc. of acetone were added. The precipitate was centrifuged off in the cold and the precipitation was repeated. From the acetone-ether solution the *acetone-soluble fraction* was obtained, 22.6 mg. of an almost white crystalline mass. The acetone-insoluble *phosphatides* formed 60.1 mg. of a light brown soft material. This fraction, as will be shown later, proved to be an extremely potent activator of blood clotting. 58.7 mg. of this fraction were dissolved in 2 cc. of ether and centrifuged for 20 minutes, whereby 1.0 mg. of an insoluble gray powder was eliminated. The ethereal solution was concentrated to 0.5 cc. and 1.25 cc. of absolute alcohol were added to the solution. The mixture was kept in the refrigerator, centrifuged in the cold, and the residue washed with a little ice-cold alcohol. The crude *cephalin* formed 16.6 mg. of a yellow wax-like material. From the mother liquor the crude *lecithin* was obtained, from which by two more precipitations a second cephalin fraction (6.3 mg.) was isolated. The crude *lecithin* formed 33.7 mg. of a light brown paste. In Experiment I the corresponding fractions were prepared in the same manner.

Acetone-Soluble Fraction—It was found that this fraction from both series of experiments consisted to a large extent of free and esterified cholesterol. We wish to thank Dr. W. M. Sperry of this University for these analyses which were carried out according to the micromethod of Schoenheimer and Sperry (10). Fraction I contained 52.6 per cent of total cholesterol, 50.3 per cent of free cholesterol. Fraction II contained 61.5 per cent of total cholesterol, 37.9 per cent of free cholesterol.

Both fractions were united and 35.4 mg. of this material were refluxed with 2 cc. of 4 per cent alcoholic potassium hydroxide for 1 hour. After isolation in the usual manner the *fatty acids* were obtained as 2.3 mg. of a brown oil and the *unsaponifiable matter* as a slightly yellow crystalline material which weighed 26.8 mg. This by precipitation with digitonin in 80 per cent alcohol was converted into the digitonide which weighed 78.8 mg., corresponding to 19.7 mg. of cholesterol. (According to the quantitative determinations given above the material contained 20.4 mg. of cholesterol.) The unsaponifiable matter, therefore, must have contained about 7 mg. of material which did not precipitate with digitonin. The digitonide was decomposed by means of pyridine

and ether (11) and 18.1 mg. of the sterol were isolated. After four crystallizations from small amounts of 90 per cent alcohol 10.4 mg. of large plates were obtained, which were identified as cholesterol. The substance melted at 146° and showed no depression of the melting point on admixture of pure cholesterol. It gave the usual cholesterol color reactions. A 0.25 per cent solution in absolute alcohol showed no selective absorption in the ultraviolet region. The amount of ergosterol or other absorbing sterols present, therefore, must have been very small. We are indebted to Mr. F. Rosebury of this Department for these measurements.

<i>Analysis</i> — $C_{27}H_{46}O$. Calculated. C 83.8, H 12.0	
386.4	Found. " 83.8, " 12.3

The aqueous phase from the saponification was neutralized with dilute barium hydroxide, centrifuged, and evaporated to complete dryness *in vacuo*. From the residue by extraction with absolute alcohol 1.8 mg. of a yellow oil were obtained. This substance, when oxidized with bromine, gave a weak reaction for dihydroxyacetone with *m*-hydroxybenzoic acid, according to the sensitive microtest for glycerol recently described by Ecgrive (12). A small amount of glycerol, therefore, seems to have been present. The reactions for carbohydrates were negative.

Cephalin—The united crude cephalin fractions (27.7 mg.) were dissolved in ether. A small amount of insoluble material (2.3 mg.) was removed by centrifugation, the ethereal solution was concentrated to 0.4 cc., and the cephalin was precipitated by slowly adding 1.5 cc. of absolute alcohol. The precipitation was twice repeated, the ethereal solution of the cephalin finally being poured into chilled acetone. The *cephalin* was obtained as an almost white wax-like substance which weighed 15.0 mg.

Analysis—Found. C 55.0, H 8.8, N 1.5, P 2.9, ash 14.7

There was not enough material available for a determination of amino N, but the substance gave a strong ninhydrin reaction (13). The mother liquors from the precipitations yielded 9.0 mg. of a light brown substance.

Lecithin—The united crude lecithin fractions (44.0 mg.) were dissolved in 0.4 cc. of absolute alcohol, and 1 cc. of a saturated

solution of cadmium chloride in methyl alcohol was added. The treatment of the precipitate with ether and chloroform, the removal of cadmium by means of a 20 per cent solution of dry ammonia in absolute methyl alcohol, and the reprecipitation with acetone were carried out according to Levene and Rolf (14). The *lecithin* finally obtained formed a slightly yellow plastic mass and weighed 16.9 mg.

Analysis—Found. C 64.2, H 11.2, N 1.9, P 3.7, ash 3.5

The ninhydrin reaction was negative. The residue from the combined mother liquors was freed of cadmium. A less pure material was obtained which weighed 26.8 mg.

Plant and Bacterial Phosphatides

Phosphatides from Soy Beans—A preparation of the mixed crude phosphatides supplied by the American Lecithin Corporation was used as starting material. The crude *cephalin* was purified by repeated precipitation from its ether solution with alcohol and from an emulsion in 10 per cent acetic acid with acetone. The final product formed a light brown, somewhat sticky powder.

Analysis—Found. C 59.7, H 10.1, N 1.4, amino N 1.5, P 3.4, ash 13.4

The preparation of the *lecithin* was carried out according to Levene and Rolf (14) with the modification that the cadmium compound before decomposition with ammonia was recrystallized twice from a mixture of 2 parts of ethyl acetate and 1 part of 80 per cent alcohol (15). The *lecithin* was light yellow and contained C 58.6, H 9.0, N 2.1, P 3.9, ash 4.0.

Cephalin from Cotton Seeds—The dark colored crude phosphatides were treated with norit. The *cephalin* prepared in the usual manner formed a brown powder.

Analysis—Found. C 54.8, H 9.2, N 1.2, amino N 1.3, P 3.4, ash 14.2

Bacterial Phosphatides—The *cephalin* and *lecithin* preparations from yeast (13) were kindly given us by Dr. L. F. Salisbury of Connecticut State College. The phosphatides from the bacillus Calmette-Guérin (16) and the diphtheria bacillus (17), which likewise were examined, have been previously described by one of us.

Experiments on Activation of Blood Clotting

Method—The experiments on the activation of blood clotting by means of various lipids were carried out with chicken plasma as substrate. The technique was essentially the same as that described in a recent communication from this laboratory (18). The measurements were carried out by adding 0.03 cc. of the activator at various dilutions in physiological saline to 0.1 cc. of plasma. The reaction temperature was 30°. The readings were repeated every 3 to 5 minutes until clotting was indicated by the immobility of the glass bead that was contained in every glass tube. All measurements were made at least in duplicate.

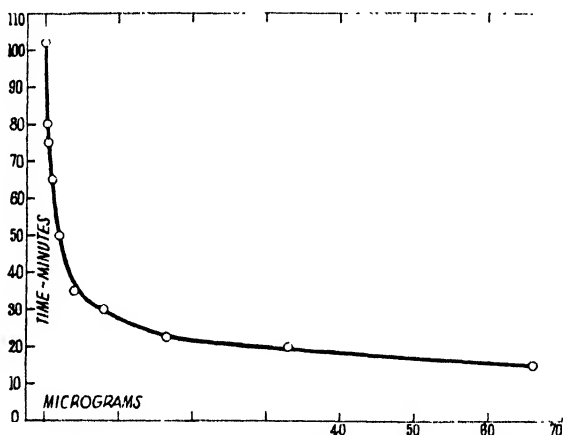


FIG. 1. Activation of plasma clotting by platelet phosphatide

The preparation of the lipid emulsions that were tested was made in the following manner: A weighed amount of the substance in a small agate mortar was dissolved by the addition of a little peroxide-free ether. The solvent was driven off by a stream of nitrogen and the evenly distributed, very thin lipid film was dried *in vacuo* for a few minutes. On treatment with physiological saline a quite stable and uniform emulsion could thus be obtained. The preparations from breast muscle which were examined could be dissolved directly in saline.

Activity of Fractions from Blood Platelets—The acetone-soluble and the chloroform-soluble fractions from platelets (Fractions 6 and 2, Table I) were entirely inactive. The mixed *phosphatide*

fraction (Fraction 3), on the other hand, proved to be a very potent activator. A typical experiment with this fraction is reproduced in Fig. 1. If, as seems convenient, a decrease in the normal clotting time of 10 per cent is taken as reference point, in this particular experiment 0.13 microgram was sufficient to effect this

TABLE II
Activation of Plasma Clotting by Platelet Phosphatides

Activator in 0.1 cc. plasma	Clotting time					
	Cephalin (crude)	Lecithin (crude)	Cephalin (purified)	Lecithin (purified)	Cephalin (mother liquor)	Lecithin (mother liquor)
<i>micrograms</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>
30	28	46	46	55	15	40
15	40	54	56	60	18	55
7.5	54	85	61	65	49	73
3.8	86	97	66	71	62	80
1.9	101	105	71	81	82	84
0.95	109	109	75	86	95	94
0.48	121	114	85			
Normal clotting time of plasma	134	134	92	92	104	104

TABLE III
Inhibitor Activity of Aqueous Platelet Extracts and of Sodium Citrate

Extract corresponding to mg. platelets per 0.1 cc. plasma...	Control	0.003	0.008	0.023	0.07	0.2	0.6
Clotting time, <i>min.</i>	106	139	155	170	230	395	>600
	104	119	135	150	188	210	410
Sodium citrate per 0.1 cc. plasma, <i>mg.</i>	Control		0.008	0.023	0.07	0.2	0.6
Clotting time, <i>min.</i>	98		130	130	>360	>360	>360

drop. The results obtained with three different plasmas were almost identical. As described above, this phosphatide mixture was separated into the crude lecithin and cephalin fractions (Fractions 4 and 5, Table I) which then were further purified. Activation experiments with these fractions and with the substances recovered from the mother liquors obtained during the purifica-

tion are reproduced in Table II. These results will be discussed later in this paper.

Aqueous extracts of the defatted platelets were examined in an endeavor to determine whether the activator had completely been removed by the organic solvents. It was very interesting to find that, contrary to expectation, these extracts possessed a marked inhibiting activity, as shown in Table III. 1 part of platelets was suspended in 50 parts of physiological saline for 5 minutes, the mixture was then centrifuged, and the clear extract was tested in various dilutions, 0.1 cc. of plasma and 0.03 cc. of the solution being used. As sodium citrate had been used ex-

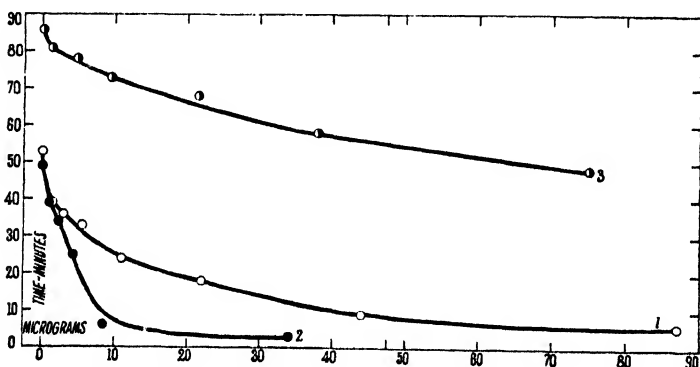


FIG. 2. Activation of plasma clotting by plant cephalins. Curve 1 represents yeast cephalin; Curve 2, soy bean cephalin; Curve 3, cotton seed cephalin.

tensively during the collection of the platelets, there was a possibility that the inhibiting action on clotting was due to sodium citrate absorbed in the cells. This does not seem to be the case. As shown in Table III, sodium citrate, even in comparatively high concentrations, affects the clotting of plasma in a different way. Sodium citrate inhibits the clotting not only of ordinary chicken plasma but also of plasma which has been activated by the addition of muscle extract (18). When tested in this manner, it is found to contain about 5 inhibitor units per mg. The aqueous platelet extract, however, is unable to prevent the clotting of plasma which contains muscle extract. It seems that this inhibitor which is extremely strong in non-activated plasma is

different from heparin. It should be mentioned that among five plasmas examined one was found which did not respond to the aqueous platelet extracts.

Activation Experiments with Other Phosphatides—The following phosphatides were found to be entirely inactive: yeast lecithin (13), soy bean lecithin, phosphatides from BCG (16) and diphtheria bacillus (17). A commercial synthetic cephalin prepared according to Grün and Limpächer (19) was likewise found inactive. A phosphatide from *Bacterium tumefaciens* (20) showed a

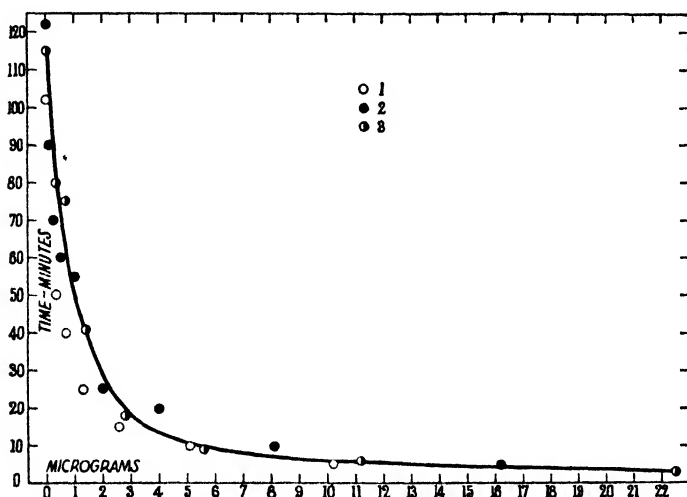


FIG. 3. Activation of plasma clotting by extract from chicken breast muscle. Experiments 1, 2, and 3 were carried out with plasma from three different chickens. The drawn out curve corresponds to Experiment 3.

slight activity. Yeast cephalin (13) and soy bean cephalin showed a very marked activating effect on plasma clotting. The cephalin preparation from cotton seeds was not quite as active. The activation of blood clotting by these three cephalins is shown in Fig. 2.

Activation Experiments with Muscle Extract—The extract from chicken breast muscle was prepared according to Fischer (21). In a large number of activation experiments very uniform results were obtained with different plasmas. The activation of plasma clotting by muscle extract is shown in Fig. 3.

DISCUSSION

Regarding the chemical composition of blood platelets, three points are particularly notable: the high amount of phosphatides and cholesterol extracted, and the low, almost negligible amount of glycerides. (We take Experiment II in Table I as the basis of our discussion.) Whether there is any essential difference in the composition of the various blood cells cannot yet be said, as our knowledge concerning the chemical constituents of erythrocytes and the various forms of white cells (with the exception of pigments and enzymes) is still very limited (compare the reviews in (22) and Boyd (23)). The comparison of results obtained by different methods cannot be of great value, as the blood cells are comparatively very unstable and may undergo permeability changes during their preparation. It furthermore should be pointed out that in most cases where the presence of substances like phosphatides or sterols has been reported in blood cells, this was only done on the basis of the phosphorus content of the extracts, or, in the case of sterols, by means of colorimetric estimation.

The experiments on the importance of platelets in blood clotting showed that there is contained in them a very potent activator of clotting which is associated with the phosphatide fraction. The activation curves (Fig. 1) obtained with this fraction are very characteristic, showing a steep fall at low concentrations and a gradual flattening out at high concentrations of the activator.

The active phosphatide fraction was separated into the cephalin and lecithin fractions and these were further purified. From the results of the activation experiments which are contained in Table II it will be seen that while all the fractions retained a certain amount of activity, the activating effect was most marked with the more soluble cephalin fraction isolated from the mother liquor. As regards the activity of the lecithin fraction, it should be emphasized that in a model experiment with a mixture of similar amounts of cephalin and lecithin from another source, and by exactly duplicating the separation procedure followed with the platelet phosphatides, an entirely inactive lecithin and a highly active cephalin could be obtained.

It is a well established fact that the activating influence of lipids on blood clotting is associated with the cephalin fraction.

(4), *i.e.* with a group of substances built up of fatty acids, glycerophosphoric acid, and aminoethyl alcohol. Whether there is one particular representative of this group which is active or whether the activity is a common property of the cephalin group cannot be said. Our present methods for the separation of phosphatides are still much too crude to permit the isolation of compounds in a state even approaching purity. It may very well be that a cephalin containing highly unsaturated fatty acids, and, therefore, more soluble in ethyl alcohol, is the real activator of blood clotting.

The fact that synthetic distearyl cephalin (19) was found entirely inactive is in harmony with the findings of McLean (24) according to whom the activity of the cephalin preparation was parallel to its degree of unsaturation.

A number of phosphatides prepared from plants and microorganisms were also tested for activity. The bacterial phosphatides were inactive. The cephalin preparations from yeast, soy beans, and cotton seeds were active, as shown in Fig. 2. The lecithin preparations from yeast and soy bean were devoid of any activity. The fact that cephalins of vegetable origin were found active tends to show that the activity is not associated with a clotting factor contained in animal tissue with which the lipids are contaminated.

The activation of clotting by muscle extract, as reproduced in Fig. 3, shows the same general picture. The activity of this substance is still more pronounced than that of the cephalin group. It is uncertain whether the mechanism of action of these two activators is the same. It must be noted that, contrary to the lipids, the muscle extract is unstable toward heating and that in some cases plasmas are found which, while responding to muscle extract, cannot be activated by cephalin.

The finding in platelets of a substance acting as a strong inhibitor of blood clotting, if borne out by repeated experiments with other platelet preparations, would prove of the greatest interest. It could serve as an explanation for the occurrence of certain blood diseases (*e.g.* hemophilia) which often have been assumed to be connected with an abnormal behavior of the blood cells. A disturbance in the permeability of the platelets for the activator or the inhibitor of clotting or the absence of either the one or the other in certain pathological cases would make for an increased bleeding or clotting tendency.

We are highly indebted to the Lederle Laboratories, Inc., Pearl River, New York, for the supply of blood cells. We wish to thank Mrs. Charlotte Breitung for assistance in some of the experiments, and Mr. W. Saschek for numerous microanalyses.

SUMMARY

1. The preparation of blood platelets from horse blood is described.

2. The platelet lipids were isolated and examined. The cephalin, lecithin, and sterol fractions are described.

3. The various platelet fractions were examined for their activating influence on plasma clotting. The phosphatide fraction was found to contain a potent activator.

4. From defatted blood platelets a substance can be extracted with water which acts as inhibitor of blood clotting. The importance of this finding is discussed.

5. The preparation of phosphatides from soy beans and cotton seeds is described. The activity in blood clotting of these substances as well as of other phosphatides of plant and bacterial origin is discussed and compared with that of tissue extracts. The cephalin fractions from soy beans, cotton seeds, and yeast were found active. This shows that the activation of clotting is the property of certain phosphatides, regardless of their origin.

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THE COLORIMETRIC DETERMINATION OF URINARY ESTRIN

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Kober (1931) was the first to suggest a colorimetric test for urinary estrin. He compared the degree of red color developed after treatment with phenolsulfonic acid in a test sample with a standard cresol red solution. Cohen and Marrian (1934) modified the Kober procedure and determined the hormone content of estrone and estriol fractions by comparison with a standard curve from a set of determinations with pure hormone, using a Lovibond tintometer for color standards. Cartland, Meyer, Miller, and Rutz (1935) have also employed the phenolsulfonic acid reagent, using both the Lovibond tintometer and a colorimeter for color comparisons of their extracts against a crystalline hormone standard. These methods have been employed for the quantitative determination of the estrins. Sala (1935) has employed the greenish fluorescence that develops after the hormones are treated with concentrated H_2SO_4 as a qualitative test for pregnancy in the mare. Chevallier, Cornil, and Verdollin (1935) have used the characteristic ultraviolet absorption at 2800 \AA . to detect estrogenic substances in human pregnancy urine extracted by alkali hydrolysis. Zimmerman (1935) has suggested that the reaction of estrone with *m*-dinitrobenzene in alkaline alcoholic solution should afford a good test. The violet color that develops occurs also with a number of other ketones. Schmulovitz and Wylie (1935) have used the orange color developed when the estrins are coupled with diazotized *p*-nitroaniline as a means of detecting these substances in human pregnancy urine, but no separation of estrone and estriol is made in their extractions. David (1934) has noted a typical blue color when estriol crystals are treated with sulfuric acid followed by arsenic acid.

We have inquired into the usefulness and limitations of certain of these procedures for the determination of urinary estrin in human and rabbit urines. Initially we determined the absorption spectrum of the colored compounds formed with crystalline estrone and estriol, using the recording spectrophotometer of Hardy (1935). Our urine extracts were divided into estrone and estriol fractions after the method of Cohen and Marrian (1934). The presumable hormone content of these fractions was determined by comparing the absorption at the color maximum (as shown by the absorption curve) by using appropriate filters of the S series in a Pulfrich photometer. For reasons which will be given later the urine samples were in every set of determinations compared with a set of crystalline hormone standards. The colorimetric assays were compared with bioassays in a number of instances.

Phenolsulfonic Acid Test—Since the exact composition of Kober's (1931) reagent is not clear, we have followed, with modifications, the methods of Cohen and Marrian (1934) and Cartland, Meyer, Miller, and Rutz (1935). Phenolsulfonic acid is made up by heating equal weights of phenol crystals and concentrated sulfuric acid at 110–120° for 15 minutes, cooling, and diluting with 2 volumes of sulfuric acid. The hormone or extracts are evaporated to dryness from an alcoholic solution under reduced pressure in a water bath at 50°.

Cohen and Marrian Test—According to Cohen and Marrian's procedure the residue is taken up in 2 cc. of the phenolsulfonic acid reagent, heated for 10 minutes in a boiling water bath, and then placed in ice water for about 1 minute. (The solution may be kept in ice at this stage for as long as 2 weeks without any material error occurring.) The solution is then made up to exactly 4 cc. with 5 per cent sulfuric acid, thoroughly mixed, cooled in ice water for about a minute, and transferred into the absorption cell. 10 minutes after mixing, the maximum color intensity develops and a reading is taken in the photometer. The maximum intensity remains constant for some 10 minutes after it is initially attained.

The absorption spectra of the colored compounds developed from crystalline estrone and estriol by this method are given in Fig. 1. It will be noted that the curves for the two substances are remarkably similar, showing two peaks, one at 510 to 512 $m\mu$

and another at 464 $m\mu$. The estriol shows a narrower, sharper band at the first peak and its absorption curve falls more sharply after the second peak. The curve for estradiol given in Fig. 1 shows a broader absorption band and a slight rise into the ultra-violet.¹ We have included the estradiol because Doisy, MacCorquodale, and Thayer (1935) find that it is probably the estrogenic ovarian follicular product.

The standard curves showing the relation between hormone concentration and color absorption were obtained by studying the

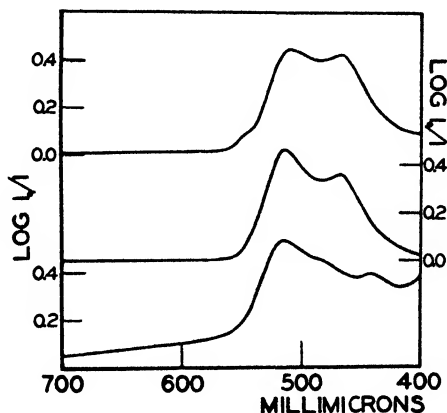


FIG. 1. Absorption spectra of the colored compounds formed with phenolsulfonic acid (Cohen and Marrian's procedure). Upper curve, estrone 33.3 micrograms; middle curve, estriol 32.3 micrograms; lower curve, estradiol 36.54 micrograms. The ordinate shows the extinction ($\log (I_0/I)$); the abscissa, wave-length in $m\mu$.

transmission through an S-50 filter on the Pulfrich photometer. Sample curves presented in Fig. 2 demonstrate that, within the concentration limits employed, the optical densities are directly proportional to the hormone concentration. The zero concentration is obtained from the reagents without added hormone, and the fact that it has a positive value indicates merely that the test reagent has itself a certain amount of color. We have been unable

¹ The estradiol curve may differ from those of estrone and estriol, either because of slight impurities present or because the crystalline compound used is in fact a mixture of two stereoisomers.

to prepare a standard curve to which we can refer day in and day out. With any given batch of phenolsulfonic acid the curves check fairly well over a limited period of time, but fresh reagent, even when made from materials from the same source, gives a curve which may differ in slope and intercept from all others (see Fig. 2). Accordingly, in making determinations of hormone

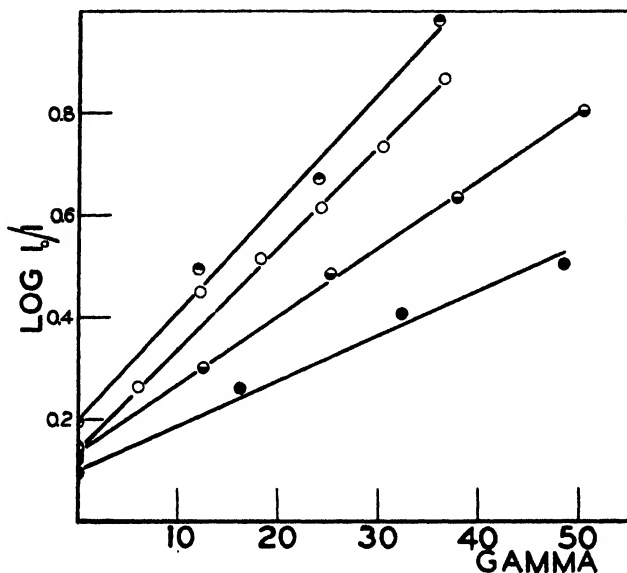


FIG. 2. Standard concentration curves for crystalline estrone and estriol, illustrating the change in slope and intercept in materials treated at different times with phenolsulfonic acid reagents. \ominus = estrone, October 31, 1935; \circ = estriol, May 16, 1936; \bullet = estrone, May 21, 1936; \bullet = estriol, December 15, 1935. The ordinate shows absorption with the S-50 filter; the abscissa, total concentration of hormone in micrograms.

content in urine extracts we have derived our urine values from a hormone concentration curve made at the same time.

The concentration values for our urine extracts were made upon at least two dilutions of the extracted material. Ordinarily the values so obtained check each other to within 5 to 10 per cent, but occasionally a difference of as much as 50 per cent is observed. In such cases several further determinations are made. Our

hormone extracts are kept in alcoholic solution in the cold; we have observed no change in the colorimetric titer after 8 months.

We have made some effort to check the delicacy of this test by making up extremely dilute solutions of both crystalline hormone and urine extracts. By making ten determinations against an equal number of blanks, it is possible to detect satisfactorily the presence of as little as 0.2 microgram of estrone or estriol. The standard errors of such sets of observations will vary from test to test, however, so that no known number of determinations can be set in advance as the necessary number.

In testing urine extracts the chief obstacle to a complete colorimetric titration is the occasional sample which develops a cloudy suspension after the addition of the 5 per cent sulfuric acid. Fortunately this occurs rarely, and in a second set of determinations the cloudiness may not appear. None the less, certain extracts will repeatedly give this cloudiness, and absorption measurements on such a suspension are of course useless.

The data giving hormone content in the two fractions of various human and rabbit urines are given in Table I. It is immediately obvious that the two rabbit urines tested show a very high titer by color test but no discoverable activity by bioassay. The reason for this difference is made clear when the absorption spectrum of rabbit urine extract is examined. Typical absorption spectra given in Fig. 3 show a fairly regular rise of absorption from the red to the violet. The sharp bands typical of the crystalline hormones are not present, although there is some indication of a slight rise in absorption in the region of the 510 and 460 $m\mu$ maxima. Inactive materials present in the extracts are responsible for the apparent hormone content.

In human pregnancy urines there are also inactive materials which give a color change in this test. This is particularly evident in the urine of earliest pregnancy, when the color test gives an estimate of about 4 times as much activity as was actually present in the estrone fractions (Urine 3). This overestimation is less marked in the estriol fractions which in later pregnancy (Urines 8 to 11) give in fact a little more activity than expected on the basis of 1.5 rat units per microgram in the color test. If there were in these bioassays 1.8 rat units per microgram of estriol, the color test would agree well with the bioassay of urines taken at 5½ months of pregnancy and later.

TABLE I

Estrone and Estrinol Content of Various Rabbit and Human Urines As Determined by Phenolsulfonic Acid Test and by Bioassay

No.	Stage of cycle	Estrone*		Estrinol*		Overestimate	
		Color test	Bioassay	Color test	Bioassay	Estrone per cent Com- plete "	Estrinol per cent Com- plete "
1	Estrus, rabbit	18.6 micrograms per cc. or 55.8 r.u.	Negative at 2.7 r.u. per cc.	31.8 micrograms per cc. or 47.7 r.u.	Negative at 2.7 r.u. per cc.		
2	Rabbit 27-30 days pregnant	150.4 micrograms per cc. or 451.2 r.u.	Negative at 2.7 r.u. per cc.	140.6 micrograms per cc. or 210.9 r.u.	Negative at 2.7 r.u. per cc.		
3†	19th day of menstrual cycle, in which conception occurred	202 micrograms or 606 r.u.	148 rat units	431 micrograms or 647 r.u.	148 rat units	309.4	337.1
4	5th day 1st mo. of pregnancy	144 micrograms or 432 r.u.	252	604 micrograms or 1812 r.u.	840	71.4	115.7
5	4 mos. pregnant	380 micrograms or 1140 r.u.	675	Cloudy	14,400	68.9	
6	4‡	472.6 micrograms or 1317.8 r.u.	450	827.5 micrograms or 1241 r.u.	685	192.8	81.2
7	5	221 micrograms or 663 r.u.	330	665 micrograms or 997.5 r.u.	600	100.9	66.3
8	5‡	Cloudy	670	975 micrograms or 1462.5 r.u.	1,780		17.9
9	6	348 micrograms or 1044 r.u.	550	1192 micrograms or 1788 r.u.	2,200	89.8	18.7
10	7	253 micrograms or 759 r.u.	Not assayed	1001 micrograms or 1501.5 r.u.	1,800		16.6
11	7-9	297 micrograms or 891 r.u.	670 (per cc.)	3600 micrograms or 5800 r.u.	8900 (per cc.)	33.0	34.8

* We assume 1 microgram of estrone = 3 rat units and 1 microgram of estrinol = 1.5 rat units.

The complete absorption spectra of the urine fractions indicate that the color due to the inactive substances is represented by

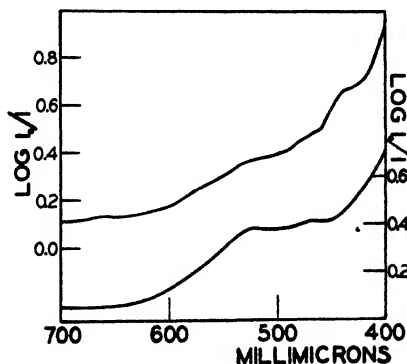


FIG. 3. Absorption spectra of rabbit urine treated with phenolsulfonic acid reagent (Cohen and Marrian's procedure). Note the rise of absorption into the violet. Upper curve, estrone fraction; lower curve, estriol fraction. The ordinate shows extinction ($\log (I_0/I)$); the abscissa, wave-length in $m\mu$.

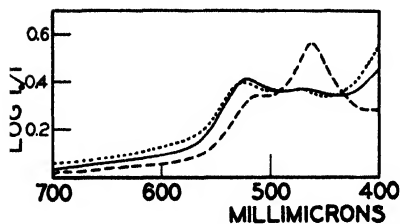


FIG. 4

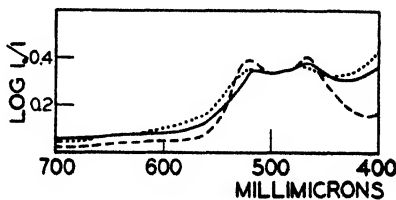


FIG. 5

FIGS. 4 AND 5. Estrone fractions (Fig. 4) and estriol fractions (Fig. 5). Absorption spectra of three human pregnancy urines treated with phenolsulfonic acid reagent (Cohen and Marrian's procedure). The dotted line represents Urine 3; the solid line, Urine 6; the dash line, Urine 11. The curves have been adjusted to give identical optical densities in the region of maximum absorption. Note that the absorption rise in the violet declines with advancing pregnancy. The ordinate shows extinction ($\log (I_0/I)$); the abscissa, wave-length in $m\mu$.

materials showing increasing absorption into the violet. This is illustrated in Figs. 4 and 5 where the curve for the earliest pregnancy urine shows the typical bands least sharply and the sharpest

rise into the violet. The estrone and estriol curves for the 7 to 9 month urines compare very favorably with the curves for the crystalline hormones (Fig. 1), showing rather sharp drops on either side of the maxima and at most only a slight rise in the violet. These urines (see Table I) gave color tests agreeing most closely with the bioassays.

Marrian and Cohen (1935) have stated that their color test is inapplicable to non-pregnancy urines. It is obvious from these data that, in using absorption methods, fairly reliable estimates of the estriol content can be had with urines of 5½ months pregnancy on, but not with earlier pregnancy urines. The estrone content will apparently be overestimated, even with the urines of later pregnancy. The content of active material can be calculated from the absorption spectra of the urine extracts by assuming that

TABLE II

Observed and Calculated Activity (in Rat Units) of Three Urine Extracts after Correction of Colorimetric Estimates (See Text)

Urine No.	Estrone		Estriol	
	Observed	Calculated	Observed	Calculated
3	148	178	148	193
6	450	495	685	605
11	670	536	890	580

the absorption by interfering substances shows a gradual increase into the violet. Since the rise in absorption by the crystalline hormones first becomes marked at 580 $m\mu$, one can make a rough interpolation on the basis of the slope of the curves of the urine extracts over the range 600 to 580 $m\mu$ and the rising portion of the curves in the violet. By subtraction at 500 $m\mu$ (where the S-50 filter transmits chiefly) the proportion due to inactive material can be estimated and a correction made. By this procedure we have estimated (Table II) the presumable active material in the three urines for which we have complete absorption spectra (Urines 3, 6, and 11). These are at best crude estimates, since we do not know the exact shape of the absorption curves of the inactive materials, and these may, in fact, differ from specimen to specimen. None the less, by this procedure the bioassays and color assays are brought into somewhat better agreement. Prac-

tically this procedure can be duplicated with the Pulfrich photometer by taking readings with the S-50 filter, the S-57, S-61, and the S-43 and interpolating on the rough absorption curve so obtained.

Test of Cartland, Meyer, Miller, and Rutz—In this test the phenolsulfonic acid reagent is made by heating equal weights of phenol crystals and concentrated sulfuric acid at 110–120° for 15 minutes, cooling, and diluting with 2 volumes of sulfuric acid. The hormone is heated with 0.2 cc. of the reagent for 2 minutes in a boiling water bath, and the mixture cooled under running water for 1 minute; 0.2 cc. of H₂O is added and heated at 125° in a glycerol

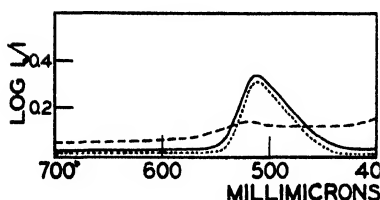


FIG. 6

FIG. 6. Absorption spectra of colored compounds formed with phenol-sulfonic acid (procedure of Cartland, Meyer, Miller, and Rutz). The solid line represents estriol, 48.4; the dotted line, estrone, 48.0; the dash line estradiol, 24.0 micrograms. The ordinate shows extinction ($\log (I_0/I)$); the abscissa, wave-length in $m\mu$.

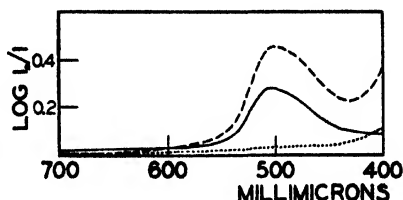


FIG. 7

FIG. 7. Absorption spectra of colored compounds formed after benzoyl chloride treatment. The dash line represents estrone, 35.23; the solid line estradiol, 36.54; the dotted line estriol, 34.08 micrograms. The ordinate shows extinction ($\log (I_0/I)$); the abscissa, wave-length in $m\mu$.

bath for 2 minutes, the mixture again cooled for 1 minute, and 0.6 cc. of water added. The absorption spectra of the hormones so treated are given in Fig. 6. It will be noted that there is only a single maximum at 514 $m\mu$ and that estradiol does not show this maximum clearly. The color fades quite rapidly in this test. This makes its use with impure extracts rather unreliable, since such extracts develop yellowish discolorations, particularly in the estriol fractions, which do not disappear until some time after the typical red color starts to fade. Furthermore the tendency for cloudiness to develop in the extracts is much increased, so that we have few reliable colorimetric determinations of our extracts by this method.

Benzoyl Chloride Test—This test represents a modification of the procedure used by Görtz (1934) for the detection of cholesterol. The solvent is removed from the hormone which is then dissolved in 2 cc. of chloroform. 1 cc. of a 40 per cent solution of zinc chloride in glacial acetic acid is added, followed by the addition of 1.5 cc. of benzoyl chloride, and the mixture is placed in a water bath at 70°. The mixture is allowed to cool with the water bath for 20 minutes, removed, and made up to 5 cc. with chloroform,

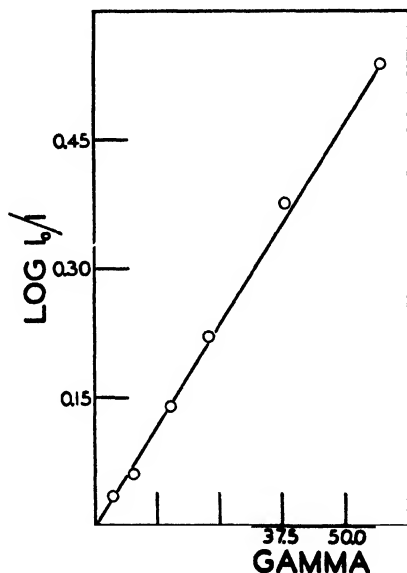


FIG. 8. A typical concentration curve for crystalline estrone in the benzoyl chloride test. The ordinate shows absorption with the S-50 filter; the abscissa, total concentration of hormone in micrograms.

and the color absorption measured with the S-50 filter on a Pulfrich photometer.

The absorption spectra of the colored compounds obtained with the hormones are given in Fig. 7. The typical absorption curve of the estrone compound is reproduced (except in the violet) by estradiol, but the estrone compound shows a greater rise at the maximum (500 to 504 $m\mu$). Estriol, on the other hand, does not give the typical estrone color with benzoyl chloride.

This test should therefore be useful in determining the estrone content of extracts, provided there are no interfering substances causing the same color. It is just as sensitive as the phenolsulfonic acid test and has the advantage that cloudy solutions are not obtained. It suffers from the disadvantage that the reagents appear to change somewhat from day to day (benzoyl chloride is markedly hygroscopic), so that a concentration curve with pure hormone must be prepared for each set of determinations. A typical calibration curve is given in Fig. 8.

In Table III are presented comparative colorimetric assays of estrone fractions of certain human and rabbit urines. The ben-

TABLE III

Comparison of Colorimetric Assays for Estrone in Certain Urine Extracts by Phenolsulfonic Acid and Benzoyl Chloride Tests

Urine No.	Case No.	Stage of cycle	Phenolsulfonic acid test	Benzoyl chloride test
			micrograms	micrograms
1	5	4½ mos. pregnant	428	473
2	8	7-9 " "	405	380
3	9	Estrus, rabbit	184	289
4	10	Rabbit 3 days pregnant	258	489
5	10	" 6 " "	248	473
6	11	" 15 " "	210	384
7	12	Hysterectomized rabbit in estrus	413	998

zoyl chloride determinations are practically identical with the phenolsulfonic acid data for human urines, but with rabbit urines benzoyl chloride tests invariably give a higher titer. The benzoyl chloride determinations with rabbit extracts are approximately twice the phenolsulfonic acid determinations, regardless of the stage of cycle of the rabbit. This implies that the rabbit urine extracts contain certain materials not present in human urines, and that these additional materials are present in constant ratio to the materials revealed by the phenolsulfonic acid test. Alternatively we may infer that there are no additional materials revealed by the benzoyl chloride test, but that the color intensity developed with benzoyl chloride is twice that of the corresponding

intensities of the hormones. This may be true, since we employ the hormones merely as our color standards for the rabbit urines which definitely lack appreciable amounts of hormone.

David Test for Estriol—The crystalline hormone is treated with 0.1 cc. of concentrated sulfuric acid, heated for 1 to 2 minutes in a boiling water bath, cooled, and while cooling dilution with 0.8 cc. of water is made. 2 small drops of 75 per cent arsenic acid are then added, the solution is heated for 1 to 2 minutes in a boiling water bath, diluted with 2.6 cc. of water, and the color absorption determined. The absorption spectrum of the blue compound developed is given in Fig. 9. The blue color occurs only with estriol and with no other hormone. The test is about half as sensitive as the phenolsulfonic acid test (compare Figs. 9 and 1)

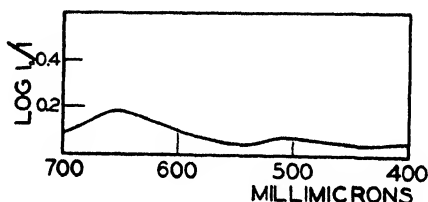


FIG. 9. Absorption spectrum of the colored compound formed by estriol in the David test; 32.3 micrograms of crystalline hormone. Compare the absorption with Fig. 1, middle curve. The ordinate shows extinction ($\log (I_0/I)$); the abscissa, wave-length in $m\mu$.

but has the advantage of being specific for estriol. Unfortunately it has proved useless with our urine extracts. Invariably a bluish cloudy suspension occurs. We have attempted to avoid the cloudiness by increasing the H_2SO_4 concentration and decreasing the H_2O , but without success. When the H_2SO_4 is increased to 0.5 cc., the typical blue color does not develop in the extracts. It is probable that with other methods of extraction or further purification this test can be extremely useful.

SUMMARY

The colorimetric determination of urinary estrin has been attempted with estrone and estriol fractions of human and rabbit urines by the use of color absorption methods. The phenolsulfonic acid test after the method of Cohen and Marrian (1934)

apparently gives reliable determinations of estriol content in human pregnancy urines of the 6th to 9th months of pregnancy. For the earlier stages of pregnancy it gives a definite overestimate owing to the presence of inactive materials that develop colored compounds absorbing at the wave-lengths of the typical hormone compounds. Estrone fractions appear to contain such materials even in late pregnancy, so that they give an overestimate of hormone content. A rough correction for the inactive materials can be made on the assumption that their colored compounds show a regular increase in absorption from the red to the violet and by subtracting these interpolated absorptions from the total absorption of the extracts. The reacting material in rabbit urines consists practically completely of such physiologically inactive materials, as indicated by the absorption spectra of the treated extracts. The phenolsulfonic acid test as used by Cartland, Meyer, Miller, and Rutz (1935) is somewhat difficult to use with impure extracts, because cloudy solutions form and color fades rapidly.

The benzoyl chloride test distinguishes estrone and estradiol from estriol. It gives a more intense color reaction with estrone than with estradiol. With the estrone fractions of human pregnancy urines it gives hormone values practically identical with those obtained by the phenolsulfonic acid test; with rabbit urine extracts the phenolsulfonic acid titer is doubled.

The David test for estriol is specific for crystalline hormone, but cannot be used with our extracts for quantitative determination because cloudy solutions invariably develop.

We are indebted to Dr. Erwin Schwenk of the Schering Corporation for the crystalline estrone and estradiol used in these experiments and to Dr. Oliver Kamm of Parke, Davis and Company for the crystalline estriol. The bioassays were made by Dr. G. V. Smith and Mrs. O. W. Smith, to whom we express our gratitude.

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CHEMICAL STUDIES OF THE SUPRARENAL CORTEX

II. THE IDENTIFICATION OF A SUBSTANCE WHICH POSSESSES THE QUALITATIVE ACTION OF CORTIN; ITS CONVERSION INTO A DIKETONE CLOSELY RELATED TO ANDROSTENEDIONE

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We have previously described the fractionation of an extract of the suprarenal gland by repeated transference from benzene to water and from water to benzene (1). Three crude fractions were thus obtained. Fraction I was the material which was most soluble in benzene, Fraction II was the least soluble in benzene, and Fraction III consisted of the material which was most soluble in water and benzene. From Fraction III Compound E was obtained to which was assigned the formula $C_{21}H_{30}O_6$. In its absorption spectrum there was a band with a maximum at 2370 Å. which showed the presence of an α, β unsaturated carbonyl group.

This substance has now assumed a position of importance, since it possesses, as far as can be determined, the same qualitative physiologic activity as cortin. Quantitatively, it is much less active than cortin. Nevertheless, the fact that it does have the physiologic qualities of cortin implies that cortin must have a closely similar structure and that a study of Compound E will also reveal the essential nature of cortin.

The physiologic activity of Compound E was determined on suprarenalectomized rats by the method of Ingle (2).¹ The mini-

¹ In this method the gastrocnemius muscles of suprarenalectomized, lightly anesthetized rats are stimulated electrically. The rats are paired, one of the pair being a control. The evaluation of the result involves the length of survival, the rate of working, and the total work performed. When sufficient cortin is supplied by injection at intervals of 12 hours, the suprarenalectomized rats perform as well as those which have the suprarenal glands intact. For purposes of standardization the minimal amount which will produce this result is determined.

mal dose required to enable the rats to work normally and to maintain the blood urea at a normal level was 1.0 mg. administered at intervals of 12 hours. It is recognized that a small proportion of cortin in the crystalline material would account for the observed activity. This possibility was eliminated as far as possible by repeated crystallizations from various solvents. Three samples, recrystallized by different means, have been tested. They have all shown the same degree of activity within the limits of error of this method. In view of the great tendency toward the formation of mixed crystals which is exhibited by the compounds found in the suprarenal gland, the possibility that cortin may have been present in these crystals is not completely eliminated, but we believe that this possibility is a remote one and are proceeding on the assumption that the structure of Compound E will furnish a clue to the structure of cortin.

The empirical formulas of the compounds isolated from the suprarenal gland and of their degradation products have long suggested that the ring structure might be identical with that found in the sterols, but direct proof was lacking. We have now found that proof with respect to Compound E. When oxidized with chromic acid, it yielded a ketone $C_{19}H_{24}O_3$ (III) which melted at 214–217° (uncorrected). This ketone was tested by Professor Koch² at the University of Chicago. His report stated that it was one-sixth to one-fourth as active as androsterone in stimulating comb-growth in the capon. Compound E itself did not show any such activity. The transformation of a compound which has the qualitative activity of cortin into a substance which has physiologic activity similar to that of androsterone definitely links cortin with the sterol ring system.

Recently Reichstein (3) has described a ketone, adrenosterone, which he obtained from the suprarenal gland and which has the property of stimulating comb growth in the capon. He (4) has also degraded another compound found in the gland to a ketone which has a similar property. This particular physiologic action furnishes strong presumptive evidence for the presence of the

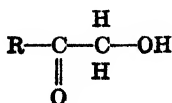
² We are greatly indebted to Professor Koch for testing the ketone and Compound E. We also wish to acknowledge our indebtedness to Professor Corner of the University of Rochester who tested Compound E for progesterone activity. At a level of 50 rabbit units it was inactive in this respect.

sterol ring system, but, since none of the compounds described by Reichstein possessed activity similar to that of cortin, the results do not furnish evidence to link cortin with such a ring system.

A study of some degradation products of Compound E has led to the conclusion that its formula is $C_{21}H_{28}O_6$ rather than $C_{21}H_{30}O_6$.

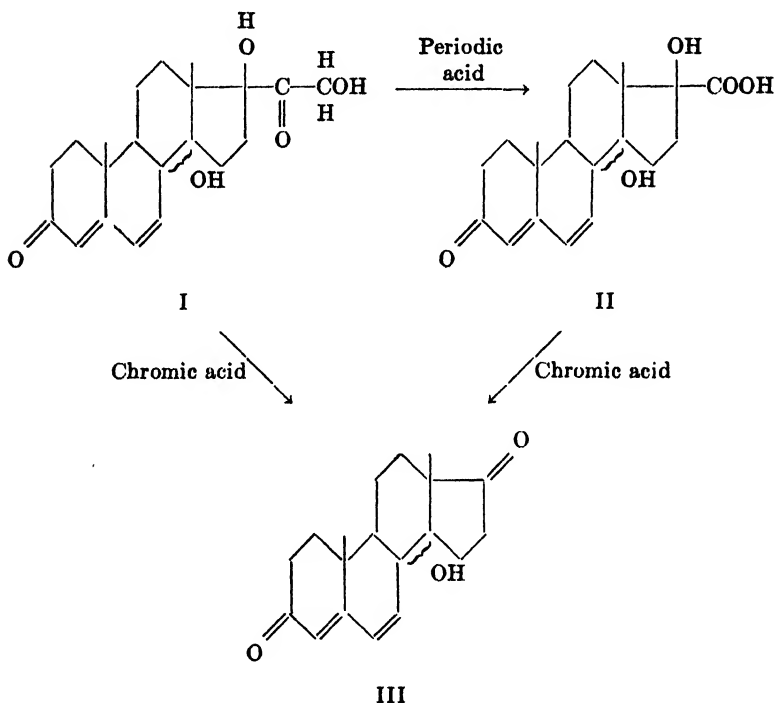
Examination of Compound E in the micro-Grignard machine immediately revealed the presence of three hydroxyl groups and one carbonyl group. Ordinarily, the methylmagnesium iodide and compound in solution were heated at 100° for 15 minutes, since it had been found that this was sufficient for the liberation of methane by the hydroxyl groups. When Compound E was so treated, 1.24 carbonyl groups were found. When the time was extended to 20 minutes, 1.40 carbonyl groups were found and after 40 minutes of heating 1.57 groups were found. This slight activity of the second carbonyl group was also shown by the formation of only the monodinitrophenylhydrazone of Compound E when a solvent which kept the mono derivative in solution and a large excess of the 2,4-dinitrophenylhydrazine were used. The presence of the second carbonyl group was demonstrated beyond question by the result of the oxidation with periodic acid. The carbonyl group which is conjugated with the double bond is indicated as the reactive group by the red color of the dinitrophenylhydrazone. The property of forming a red dinitrophenylhydrazone is also found in the acid (II) which has only the one carbonyl group and in the ketone (III) which forms only the monodinitrophenylhydrazone in the cold. When heated 25 minutes with the Grignard reagent, the ketone (III) showed 1.43 carbonyl groups and one hydroxyl group.

The formation of the ketone $C_{19}H_{24}O_3$ (III) by oxidation of Compound E with chromic acid showed the presence of a side chain of 2 carbon atoms, each of which bore an oxygen atom. In order to determine the functions of these oxygen atoms the substance was oxidized with periodic acid. An acid $C_{20}H_{28}O_5$ (II) (m.p. $263-265^\circ$) and formaldehyde were the products. Clutterbuck and Renter (5) have shown that these products could come only from the grouping



Further oxidation of the acid (II) with chromic acid yielded the ketone (III).

In view of these facts and the physiologic findings we suggest (I) as a provisional formula for Compound E.



The resistance of the one hydroxyl group to oxidation with chromic acid suggests that it occupies a tertiary position. The evidence for the second double bond rests on the analyses of the acid (II) and the ketone (III). It is tentatively placed in conjugation with the known double bond, since Compound E does not give a color with tetranitromethane.

Since we have chosen to number our degradation products, the acid (II) will be termed Acid 5 and the ketone (III) Ketone 4.

Compound E remained unchanged in solutions of pH about 2.5 to pH 8. However, when it was treated with 1 mole of sodium hydroxide, it was quickly converted to an acid. A similar change may explain the ease with which cortin is destroyed by alkalis.

There was described in our first paper (1) an acid, $C_{20}H_{28}O_4$ (Acid 1), which now appears to be related to Compound E in its structure. The absorption spectrum of Acid 1 also has the band with a maximum at 2380 \AA ., which is indicative of an α, β unsaturated carbonyl group. Analysis of the 2,4-dinitrophenylhydrazone of Acid 1 confirmed the formula $C_{20}H_{28}O_4$.

Work is now under way to determine the relationship of Compound E to Acid 1 and to confirm by chemical means the physiologic evidence of structure. It is anticipated that Acid 1 and Acid 5 can be converted into etiocholanic or etioallocholanic acid if our conclusions are correct.

EXPERIMENTAL

Isolation of Compound E—Progress toward the isolation of Compound E was checked by determinations of the specific rotations of the various fractions. The specific rotation of the crude Fraction III was usually above 220° in benzene. Fractionation was continued until this value was $+270^\circ \pm 4^\circ$ in the same solvent. It has not been possible to increase the specific rotation above this value which was given previously as the specific rotation of Compound E. In the later work the solvent was 95 per cent alcohol. In this solvent $[\alpha]_{5461}^{25} = +248^\circ \pm 4^\circ$ ($c = 0.1$ to 0.2).

The essential details of the procedure for the isolation of the material which was used for the rat test of cortical hormone activity will be described, but in the later work crystallization from absolute alcohol has been used almost exclusively, since this was found to be a very efficient means for the isolation of Compound E.

The crude fraction weighed 567 mg. and its specific rotation was 223° in benzene. It was crystallized from 175 cc. of water by evaporation of the acetone from an acetone-water solution. $[\alpha] = +244^\circ$. Crystallization from 40 cc. of benzene did not change the specific rotation appreciably. It was crystallized a second time from 100 cc. of water and then from 10 cc. of methanol. The product weighed 174 mg. and $[\alpha] = +274^\circ \pm 5^\circ$ in benzene. This was tested on the rats.

88 mg. were recrystallized from 2 cc. of absolute alcohol. The weight was 49 mg. and $[\alpha] = +268^\circ \pm 4^\circ$ in benzene. This material possessed the same degree of physiologic activity as that from which it was derived.

The third sample for the physiologic test was obtained from the filtrate from the 174 mg. above. The filtrate (10 cc.) was diluted with 20 cc. of water and cooled in the ice box. The crystals (120 mg., $[\alpha] = +240^\circ$) were dissolved in 3 cc. of methanol. Addition of dry ether caused the crystallization of 62 mg. $[\alpha] = +270^\circ \pm 4^\circ$ in benzene. A portion (24.5 mg.) of this was used in an attempt to prepare an acetone derivative by the usual method of shaking with acetone and anhydrous copper sulfate. The copper sulfate was removed and the shaking continued for 2 hours with anhydrous potassium carbonate. After removal of the potassium carbonate and acetone the residue was recrystallized from benzene. The product (11 mg.) melted at $207\text{--}215^\circ$ (uncorrected) with decomposition and $[\alpha]_{5461}^{25} = +272^\circ \pm 7^\circ$. It was evidently unchanged Compound E. It was tested on the rats and found to have the same activity as the two other samples.

Formula of Compound E—Two preparations of Compound E were analyzed. The first preparation was obtained by allowing the acetone to evaporate slowly from an acetone-water solution of material which had a specific rotation of 270° in benzene. There was no change in specific rotation after recrystallization. The second preparation was obtained by recrystallization from absolute alcohol of material which also showed a specific rotation of 270° in benzene.

$C_{21}H_{30}O_5$.	Calculated.	C 69.57, H 8.35
$C_{21}H_{28}O_5$.		" 69.96, " 7.83
	Found. Preparation I.	" 69.83, 70.04, H 8.18, 8.19
	" II.	" 70.02, " 8.08

Although the values for carbon agree well with that calculated for $C_{21}H_{28}O_5$, the values for hydrogen tend to be rather high. However, when the analyses of the acid and ketone derived from Compound E are taken into account, the formula $C_{21}H_{28}O_5$ is the more likely one.

Determination of Active Hydrogen Atoms and Carbonyl Groups—Preparation I was used for these determinations (Table I).

Oxidation of Compound E with Periodic Acid. (a) *Isolation of Acid 5*—207 mg. (0.000575 mole) of Compound E were dissolved in 90 cc. of alcohol. To this were added 49.4 cc. of an $m/80$ solution of periodic acid and 2 cc. of 5 N sulfuric acid. This amount of

periodic acid will liberate from potassium iodide a quantity of iodine equivalent to 49.4 cc. of 0.1 N thiosulfate. After having stood 24 hours at room temperature, the alcohol was removed under reduced pressure. The distillate was received under 25 cc. of Brady's reagent. The aqueous residue was filtered from 140 mg. of crystalline product and extracted three times with ethyl acetate. The water solution was titrated with thiosulfate after the addition of potassium iodide. The iodine which was liberated required 37.3 cc. of 0.1 N thiosulfate. The reaction had resulted in the consumption of 0.000605 gm. atom of oxygen, which is 1.05 atoms for each molecule of Compound E. The aqueous solution was reserved for the isolation of formaldehyde.

The ethyl acetate solution was washed with a solution of sodium carbonate. There remained in the ethyl acetate 3 mg. of non-acid.

TABLE I

Estimation of Active Hydrogen Atoms and Carbonyl Groups in Compound E

Sample	Time of heating	Active hydrogen atoms found	Carbonyl groups found	No. of groups found	
				OH	CO
<i>moles</i> $\times 10^3$	<i>min.</i>	<i>moles</i> $\times 10^3$	<i>moles</i> $\times 10^3$		
4.02	15	12.22	5.0	3.04	1.24
4.06	20	12.22	5.68	3.01	1.40
4.06	40	13.12	6.38	3.25	1.57

The washings were acidified and extracted with ethyl acetate. The ethyl acetate was dried with sodium sulfate and distilled. The crystalline residue weighed 57 mg. Since it was slightly discolored, it was shaken in acetone solution with a small amount of charcoal. After filtration and evaporation of the acetone this fraction was combined with the first and recrystallized from ethyl acetate. The first two crops of crystals weighed 100 mg. and 40 mg. and melted with marked decomposition at 263–265° (uncorrected) after some darkening and softening at about 250°. [$\alpha_{D}^{25} = +217^\circ \pm 3^\circ$ (0.17 per cent in alcohol).]

$C_{20}H_{20}O_5$. Calculated.

C 68.92, H 8.10

$C_{20}H_{20}O_5$.

" 69.32, " 7.57

Found. Preparation I.

" 69.18, " 7.65

"

II. " 69.31, " 7.72

Acid 5 formed a red 2,4-dinitrophenylhydrazone which could not be obtained crystalline.

(b) *Identification of Formaldehyde*—When the alcohol was removed from the mixture of distillate and Brady's reagent (a saturated solution of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid), 35 mg. of yellow crystals separated. The solution which had been titrated with thiosulfate was also treated with Brady's reagent. This yielded 65 mg. of a yellow 2,4-dinitrophenylhydrazone. The crude material melted at 158–160°. After crystallization from alcohol the melting point was 161–163° (uncorrected). Admixture with an authentic specimen of formaldehyde 2,4-dinitrophenylhydrazone did not cause a depression of the melting point. The known specimen melted at 163–165° (uncorrected).

$C_7H_6O_4N_4$. Calculated. C 39.98, H 2.88; found, C 40.07, H 3.38

The 100 mg. of dinitrophenylhydrazone correspond to 14.3 mg. of formaldehyde, which is a recovery of 83 per cent of the amount calculated.

Oxidation of Compound E with Chromic Acid. Ketone 4—To 100 mg. of Compound E dissolved in 7 cc. of acetone were added 5 cc. of a 1.95 N solution of potassium dichromate, 13 cc. of N sulfuric acid, and 5 cc. of water. After 22 hours at room temperature the acetone was removed by distillation under reduced pressure. The aqueous residue was extracted with ether. After the ether was washed with a solution of sodium carbonate, 72 mg. of non-acid remained. This was again treated in 5 cc. of acetone with one-half the amounts of the reagents used the first time. After 20 hours at room temperature 39 mg. of crystalline product had separated. This was decolorized in acetone solution with charcoal; 5 cc. of water were added and the acetone evaporated. The 36 mg. so obtained were recrystallized from dilute alcohol and dried for 2 hours at 110° and 0.1 mm. M.p. 214–217° (uncorrected) with decomposition.

$C_{11}H_{14}O_3$. Calculated. C 75.95, H 8.06, 1 OH 5.67, 2 CO 18.3
Found. " 75.83, " 8.22, " 5.36, " 13.2

Oxidation of Acid 5 with Chromic Acid. Ketone 4—43 mg. of Acid 5 were oxidized with chromic acid as described in the preced-

ing experiment. The non-acid product amounted to 35 mg. Upon recrystallization from dilute alcohol 19 mg. of needles were obtained. M.p. 214–217° (uncorrected) with decomposition. $[\alpha]_{5461}^{25} = +364^{\circ} \pm 5^{\circ}$ (0.18 per cent in absolute alcohol).

Dinitrophenylhydrazone of Ketone 4—9 mg. of the ketone in 6 cc. of alcohol were treated with 2.5 cc. of Brady's reagent. Red crystals slowly separated. They weighed 14 mg. and, after recrystallization from dilute acetic acid melted at 254–256° (uncorrected) with decomposition.

$C_{22}H_{18}O_6N_4$.	Calculated.	C 62.47, H 5.88
	Found.	" 62.12, 62.27, H 5.97, 5.98

Effect of Alkali on Compound E—To 58 mg. (0.00016 mole) of Compound E in 24 cc. of alcohol were added 1.50 cc. of 0.1 N sodium hydroxide. A blank was run simultaneously. After 20 minutes the solutions were titrated with 0.1 N sulfuric acid. It was found that the Compound E had used 1.15 cc. of 0.1 N base. The solution was again made alkaline and extracted with ether. It was then acidified and further extracted. This ether extract contained 45 mg. of acid, which is equivalent to 1.25 cc. of 0.1 N acid, assuming the molecular weight is 360, the same as that of Compound E.

There was no change in the specific rotation (235° in 50 per cent alcohol) when a solution of 15 mg. in 5 cc. of alcohol and 5 cc. of phosphate buffer of pH 8 was allowed to stand overnight. Similarly, there was no change in a solution of 15 mg. in 10 cc. of 50 per cent alcohol which contained 0.5 cc. of 0.1 N sulfuric acid.

Dinitrophenylhydrazone of Acid 1—The 2,4-dinitrophenylhydrazone was prepared from 8 mg. of the acid. This was dissolved in 5 cc. of methanol and treated with 2 cc. of Brady's reagent. The crude product was amorphous but crystallized from dilute alcohol in needles and then weighed 10 mg. M.p. 270° (uncorrected) with decomposition.

$C_{20}H_{16}O_7N_4$.	Calculated.	C 61.14, H 5.92, N 10.98
	Found.	" 61.35, " 5.99, " 11.17

Absorption Spectrum of Acid 1—This was determined in alcohol solution with the sample described previously. A Gaertner quartz spectrograph and rotating sector were used. One absorp-

tion band with a maximum at 2380 Å. was found. The molecular absorption coefficient at this wave-length was 20,220 (concentration 0.000363 mole per liter, length 0.075 cm.).

SUMMARY

From the suprarenal gland there has been isolated a trihydroxydiketone, $C_{21}H_{28}O_6$, Compound E, which has a physiologic activity similar to that of cortin in quality although not in quantity. This substance has been oxidized to a ketone, $C_{19}H_{24}O_3$, which stimulated comb growth in the capon. Since this is presumptive evidence that the degradation product of the trihydroxydiketone contains the sterol ring system, cortin-like activity is associated with the sterol ring system in the trihydroxydiketone. The hypothesis is proposed that cortin must also contain such a ring structure. A provisional formula for Compound E is suggested.

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THE SYNTHESIS OF DI-N-METHYLHOMOCYSTINE AND N-METHYLMETHIONINE AND A STUDY OF THEIR GROWTH-PROMOTING ABILITY IN CONNECTION WITH A CYSTINE-DEFICIENT DIET

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Considerable interest has recently been shown in the metabolism of N-methylamino acids. Gordon and Jackson (1) have reported, for example, that *dl*-amino-N-methyltryptophane may be utilized for growth in lieu of tryptophane and Fishman and White (2) have demonstrated that *dl*-amino-N-methylhistidine may serve for histidine in the diet of the white rat. These methylamino acids, as pointed out by Krebs (3), are probably oxidatively deaminized by the body to the corresponding keto acids. In fact, Keilin and Hartree (4) found that *d*-N-methylalanine could be deaminized by *d*-deaminase to pyruvic acid. The ability of these methylamino acids to support growth is due no doubt to the conversion of the keto acids formed from them to the amino acids, for it has already been shown that indolepyruvic acid (5, 6) and imidazolepyruvic acid (7) are capable of supporting growth in place of the corresponding amino acids.

On the basis of these considerations it was felt that it would be of interest to study the availability of the N-methyl derivative of homocystine and to compare its behavior with that of N-methylmethionine. Such a study would bring to bear at least indirect evidence on the utilization of the corresponding keto acids of homocystine and methionine. It would also afford a further comparison of the metabolism of these two amino acids and their derivatives as well as furnish further information concerning the behavior of methylamino acids in the body.

For the preparation of the methylamino acids which were to be used in this study, direct synthesis seemed preferable to the methylation of the amino acids themselves. For such a synthesis benzylthioethylmalonic acid, synthesized by Patterson and du Vigneaud (8) as an intermediate in the synthesis of homocystine, appeared to be an attractive starting material. This compound was prepared according to the directions given in the communication referred to and was then brominated. The bromomalonic acid derivative was treated with methylamine, carbon dioxide split out of the resulting product, and the S-benzyl-N-methylhomocysteine isolated. This compound served as a common precursor for both the di-N-methylhomocystine and the N-methylmethionine. The former was prepared by reduction of the benzylmethylhomocysteine in liquid ammonia with metallic sodium and subsequent oxidation of the resulting sulfhydryl compound according to the directions for the conversion of benzylhomocysteine to homocystine (9). The methylmethionine was formed by the reduction of the benzylmethylhomocysteine with sodium in liquid ammonia followed by the addition of methyl iodide to the liquid ammonia solution of the reduced compound. The methylmethionine was somewhat more difficult to purify than the homocystine analogue.

With the methylhomocystine and methylmethionine obtained by the above procedures the study of the ability of these methylamino acids to support growth of animals on a cystine-deficient diet was undertaken. As shown in Chart I both of these methylated amino acids were capable of serving in lieu of cystine under these conditions. Furthermore, the rate of growth obtained was as rapid as that obtained with an equivalent amount of cystine. The utilization of these N-methylamino acids proceeds no doubt through a deamination to the keto acid, as discussed earlier in this communication in connection with other methylamino acids. We would therefore infer from these experiments that the keto acids corresponding to homocystine and methionine can be utilized by animals on a cystine-deficient diet.

If the keto acid corresponding to methionine can be utilized, the possibility arises that perhaps the first step in the metabolism of methionine is an oxidative deamination rather than the demethylation postulated by ourselves (10-12) and others (13, 14). The

demethylation of the keto acid might then take place to give the keto acid corresponding to homocysteine.

These results with the N-methyl derivatives of methionine and

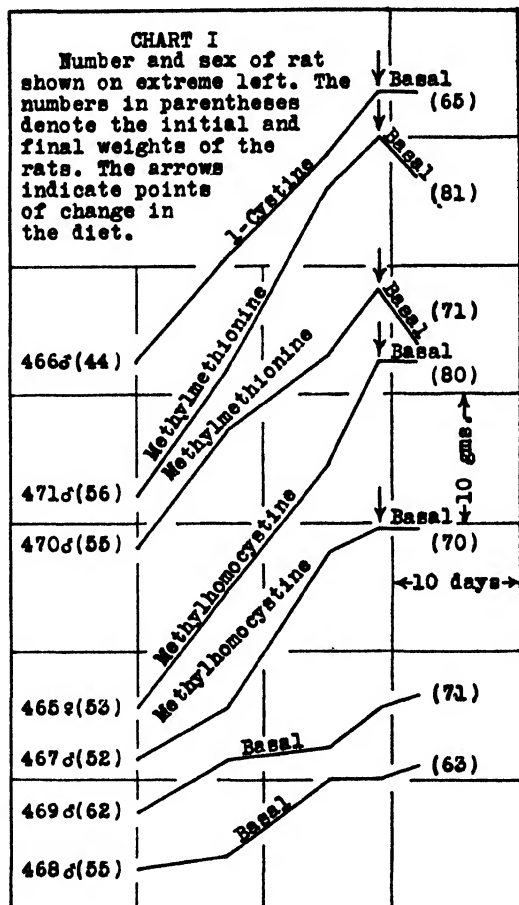


CHART I. Growth of animals on a cystine-deficient diet supplemented with di-N-methylhomocystine or N-methylmethionine. Litter I.

homocystine offer another instance in which the metabolic behavior of these two amino acids and their derivatives parallel one another in the normal animal, other instances having been brought out in previous communications (11, 12, 15).

EXPERIMENTAL

Preparation of S-Benzyl-N-Methylhomocysteine—60 gm. of benzylthioethylmalonic acid were dissolved in 500 cc. of absolute ether. A few drops of dry bromine were added with stirring to the solution cooled in an ice bath. When this bromine had reacted, 12 cc. of dry bromine were introduced during an interval of 10 minutes. The reaction mixture was immediately poured into 400 cc. of 33 per cent methylamine. After the mixture was allowed to stand for 2 days, the water layer was separated from the ether layer. The methylamine was removed from the water layer by distillation and was condensed in a receiver cooled in a bath consisting of a mixture of trichloroethylene and solid CO_2 . The aqueous solution remaining after removal of the methylamine was acidified with 100 cc. of concentrated HCl and was refluxed for 5 hours. After the mixture had cooled slightly it was neutralized with concentrated NH_4OH . It was then allowed to cool to room temperature. The precipitate was filtered and washed thoroughly with water and then with 95 per cent ethyl alcohol. The dry product, sufficiently pure for the next step, weighed 28.5 gm., which represented 50 per cent of the theoretical yield. For analysis the substance was recrystallized by solution in dilute HCl , filtration through norit, and reprecipitation with NH_4OH . A mass of colorless silky needles resulted. The product melted at $220\text{--}222^\circ$ (corrected) and microanalyses showed that it had the following composition.

$\text{C}_{12}\text{H}_{17}\text{O}_2\text{NS}$.	Calculated.	C 60.20,	H 7.16,	N 5.86,	S 13.40,	CH_3 6.28
	Found.	" 60.36,	" 7.20,	" 5.74,	" 13.54,	" 6.78

Preparation of Di-N-Methylhomocysteine—To 75 cc. of liquid NH_3 in a large test-tube suspended in a cooling bath of solid CO_2 and trichloroethylene, 7.6 gm. of S-benzyl-N-methylhomocysteine were added. A few glass beads were put in the tube to facilitate the solution of the material when the tube was shaken. After all of the substance had dissolved, sodium was introduced in small portions until a slight excess was indicated by the blue color. The ammonia was then allowed to evaporate and the residue was dissolved in 40 cc. of water. After the solution was neutralized to phenolphthalein with HCl , a stream of air was passed through it in the presence of a little FeCl_3 . When the test for the sulphydryl

group with nitroprusside became negative, the mixture was neutralized to litmus with HCl and was filtered. The precipitate was treated with 50 cc. of warm water containing enough NaOH to dissolve the compound. This solution was filtered through a little norit and was neutralized while hot with HCl. The solution upon cooling became a jelly-like mass of silky needles. These were filtered and washed with water and alcohol. A small amount of additional material was obtained from the mother liquor by concentration. The dry product weighed 3.8 gm., which amounted to 80 per cent of the theoretical yield. The di-N-methylhomocystine was found to be more soluble in water than homocystine. A sample, recrystallized from water, decomposed at 257–260° (corrected) with rapid heating and gave the following analysis.

$C_{10}H_{20}O_4N_2S_2$.	Calculated.	C 40.50,	H 6.80,	N 9.46,	S 21.64,	CH ₂ 10.14
	Found.	" 40.50,	" 6.67,	" 9.40,	" 21.94,	" 9.64

Preparation of N-Methylmethionine—21.5 gm. of S-benzyl-N-methylhomocystine in 300 cc. of liquid NH₃ were treated with 5.4 gm. of sodium and 6 cc. of methyl iodide were slowly added. After the ammonia had been allowed to evaporate, the oily residue was treated with water and was extracted with ether to remove toluene and dibenzyl. The aqueous layer was neutralized with 24.7 cc. of 5.87 N HI, an amount equivalent to the sodium used. This mixture was concentrated to a viscous syrup *in vacuo*, 200 cc. of acetone were added, and the concentration repeated. After two more concentrations with acetone the residue was dissolved in 500 cc. of hot acetone and the solution was set in the ice box overnight. After filtration the mother liquors were diluted to 1 liter with acetone and were set in the ice box for 1 week, at the end of which the precipitate was filtered. The combined precipitates were dissolved in the minimum amount of boiling water and cooled. The precipitate was filtered and the filtrate was treated with an equal volume of alcohol. After standing in the ice box overnight, the mixture was filtered. A small additional amount of pure material was obtained from reworking all of the final mother liquors. The total yield was 8 gm., which was 55 per cent of the theoretical amount.

The pure N-methylmethionine crystallized from water in large

thin rectangular plates melting at 255–257° (corrected). Microanalyses showed that the compound had the following composition.

$C_8H_{11}O_2NS$. Calculated. C 44.13, H 8.03, N 8.58, S 19.65, CH_3 18.41
 Found. " 44.14, " 7.82, " 8.51, " 19.82, " 18.70

Growth Experiments—Two litters of rats were used for the investigation. The same procedure which has been used for similar studies in this laboratory was followed (15) and the details will

TABLE I
Food Consumption

Rat No. and sex	Days	Daily supplement to basal diet	Daily food consumption
			gm.
468 ♂	1–12	20 mg. <i>l</i> -cystine	4.2
	12–34		6.0
469 ♂	1–12		5.7
	12–34		7.9
466 ♂	1–12		4.0
	12–31		6.4
	31–34		6.7
465 ♀	1–12		4.7
	12–31		8.3
	31–34		6.7
467 ♂	1–12		4.2
	12–31		6.6
	31–34		8.0
470 ♂	1–12		4.0
	12–31		6.6
	31–34		7.3
471 ♂	1–12		4.7
	12–31		7.4
	31–34		6.0

therefore not be given here. The basal diet had the following composition: casein 6.0, dextrin 33.0, sucrose 15.0, agar 2.0, salt mixture (Osborne and Mendel (16)) 4.0, lard 19.0, cod liver oil 5.0, and milk vitamin concentrate (Supplee *et al.* (17)) 16.0. The milk vitamin concentrate was increased from the 12 per cent used in previous work to 16 per cent as a new supply of milk vitamin concentrate was received and the larger quantity was found necessary. The dextrin was correspondingly decreased. All the ani-

imals were placed on the basal diet for a fore period of 12 days. At the end of this period two dextrin pills containing 24.7 mg. each of di-N-methylhomocystine, twice the equivalent amount of sulfur in 10 mg. of cystine, were given daily to Rats 465 and 467, one in the morning and one at night. Two pills containing 27.2 mg. of N-methylmethionine each, the equivalent in sulfur of the methylhomocystine supplement, were given daily to Rats 470 and 471. Rat 466 was given two pills daily containing 10 mg. of *l*-cystine in each pill. Rats 468 and 469 were kept on the basal diet during the entire experimental period. The methylamino acids were given in twice the equivalent amount of *l*-cystine, since they were optically inactive. After 19 days the supplements were withdrawn and all of the animals of the litter were kept on the basal diet. The growth curves are given in Chart I and the food consumption is given in Table I. With the second litter of rats employed, two animals received methylhomocystine, two methylmethionine, and two were kept on the basal diet. The results obtained with this litter were almost identical with the results with the first litter. Because of the great similarity in the data the growth curves for the second litter have not been presented in order to conserve space.

The authors wish to thank Mr. C. Rodden, microanalyst of this laboratory, for carrying out the microanalyses.

SUMMARY

Di-N-methylhomocystine and N-methylmethionine have been synthesized from benzylthiolethylmalonic acid.

It has been shown that both of these methylamino acids are able to support the growth of animals on a cystine-deficient diet. The behavior of the keto acids corresponding to homocystine and methionine which might be inferred from these results has been pointed out.

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CHEMICAL STUDIES ON PROLAN (FROM URINE OF PREGNANCY)

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The purposes of a chemical study of prolan were essentially the same as those which prompted us to study two other protein hormones, insulin (1) and the pituitary gonadotropic hormone (2).

Preparation of Prolan—All of the studies were made upon the same batch of prolan, which was prepared from urine of pregnancy by a series of fractional alcohol precipitations. At pH 4.0 and pH 8.5, 50 per cent alcohol-insoluble fractions were removed. The final product, washed with alcohol and finally with ether, was air-dried, powdered, and stored in a vacuum desiccator over P_2O_5 .

The absence of estrogenic substances (and their male analogues) was assured by negative responses in castrated rats (four females and four males) dosed with 100 and 400 times the minimum luteinizing dose.

Assay—22 to 23 day-old albino rats were dosed subcutaneously once daily for 4 days and sacrificed on the 6th day. The standardization of the prolan was performed at four dosage levels, four criteria serving as objective measures. The data are given in Table I. The data were rejected if the body weight of the males the 6th day was not in the range 45 to 60 gm., or if the body weight of the females was not in the range 40 to 55 gm. The means and standard deviations of the mean are given for six males and six females at each dosage level. Since many of the reactions were studied in 2 per cent $NaHCO_3$ solution, the standardization was repeated with the same number of animals for prolan dissolved in 2 per cent $NaHCO_3$. The results are not recorded, as they agreed with the control standardization within once the standard deviation of the mean, with the exception of

seminal vesicle weights for the high dosage (1 mg.). The seminal vesicle weights averaged 25 ± 2 mg.

It will be noted that the four methods of assay possess the same degree of accuracy provided the correct dosage is used. In an assay with six animals, a 25 per cent difference would be without significance. Whenever the results of the experiment were doubtful, a repetition with litter mates as controls was initiated (3).

Reactions—The amounts of prolan and of various chemical reagents, the reaction volume, and other reaction conditions are given in Table II. Unless otherwise stated, the reaction was permitted to take place during 1 hour at room temperature. The reaction mixture was then kept refrigerated, except when used for dosage.

In studying the effect of concentrated sulfuric acid, the sulfuric

TABLE I
Standardization of Prolan

Total dose	Weight of ovaries	Weight of seminal vesicles	Weight of prostate gland	Corpora lutea
mg.	mg.	mg.	mg.	
1.0	32 ± 1	31 ± 1	139 ± 13	+
0.4	24 ± 2	26 ± 2	105 ± 5	+
0.1	15 ± 2	19 ± 1	95 ± 6	+
0.04	12 ± 2	14 ± 1	70 ± 5	+—
0.00	12 ± 2	9 ± 1	53 ± 3	—

acid solution was kept at -10° for 15 minutes; 4 gm. of ice were then added, followed by 200 mg. of NaHCO_3 . The final pH of the reaction mixture was 6.0.

Alcohol was removed from the CS_2 reaction mixture *in vacuo* at 40° .

The diazobenzene sulfonate was permitted to react with the prolan in a NaHCO_3 buffer solution. The reaction pH was 7.4. Excess of diazo reagent was coupled with 20 mg. of cresol.

In the studies on insulin and pituitary gonadotropic preparation, excess of reagent was separated from reaction products in most cases by processes known to separate the original material. It was necessary to assume that the properties of the reaction products were not sufficiently changed to interfere with their recovery. In the present studies no separation of the reaction products from

TABLE II
Effect of Various Reagents upon Prolan

Prolan	Reaction mixture	Reagent and reaction environment	Recovery as measured by				No. of test rats
			Seminal vesicle weight	Prostate weight	Ovary weight	Luteal response	
mg.	cc.		per cent	per cent	per cent	per cent	
12	3	0.1 N HCl; 24 hrs., pH 2.0	30		20	5	6
12	3	0.1 " " 48 " " 2.0	<10		5	5	6
12	6	0.1 " " 1 hr., 40°	20	10	25	10	11
7	3.5	0.1 " " $\frac{1}{2}$ " 40°	40	15			6
5	5	50 mg. AcOH			100		6
10	0.05	90 " concentrated H ₂ SO ₄ ; 15 min., -10°	5	5	5	>5	7
10	10	10 mg. NaNO ₃ , 60 mg. AcOH; pH 4.0, 0°	100	100	100		8
7	7	20 mg. HCHO; pH 4.5	80	100			6
7	7	20 " " 140 mg. NaHCO ₃	100	100	75		6
6	6	40 " AcH, 120 " "	100		100		6
8	8	30 " (Ac) ₂ O; pH 4.0	75	75	75		10
12	6	40 " " 120 mg. NaHCO ₃	<5		10	5	10
8	4	40 " BzCl, 80 " "	<5				4
8	8	30 " (Me) ₂ SO ₄ , 80 mg. AcONa--3H ₂ O; pH 5.0	40	40	50		19
12	6	30 mg. (Me) ₂ SO ₄ , 120 mg. NaHCO ₃					
1.6	8	40 " " 160 " "	70		60	>50	14
9.5	9.5	45 " β -naphthoquinone sulfonate; pH 6.5	50	30	50		9
4	4	40 mg. β -naphthoquinone sulfonate, 80 mg. NaHCO ₃	<5	<10	<10	<5	6
6	6	0.3 cc. 0.01 N I (in KI); pH 3.5	100	100			3
6	6	0.3 " 0.01 " " " " 6.0	40	40			6
5	5	2.5 " 0.01 " " " " 6.0	40	40			6
7	7	3.0 " 0.01 " " " " 140 mg. NaHCO ₃ ; pH 8.5	5	0			7
6	6	40 mg. PhNCO, 120 mg. NaHCO ₃	20		25	20	7
8	8	30 " CS ₂ , 80 mg. NaHCO ₃ , 5 cc. EtOH; 2 hrs.	30		25		8
5	5	15 mg. ICH ₂ COOH, 100 mg. NaHCO ₃	100		100		6
7	7	15 mg. diazobenzene sulfonate; pH 7.4	5	5	30	10	13
7	7	6 mg. NaCN (neutralized); pH 6.5	100	100			6
5.5	5.5	100 mg. H ₂ O ₂ , 100 mg. NaHCO ₃	5	<5	<10		12
5.5	5.5	100 " " 55 " AcOH	80	20	80		12
10.0	10.0	2 cc. 0.01 N KMnO ₄ , 50 mg. AcOH	<5	<5	10	<5	16

excess reagent was made. The amounts of prolان required for physiological assay are so minute, because of the high physiological activity, that excess of reagent did not interfere with the assay.

Methylation of Pituitary Gonadotropic Preparation—The response of prolان to the various reagents studied was analogous to the response obtained with the pituitary gonadotropic preparation as previously reported, with the exception of the reaction with dimethyl sulfate. Methylation of the pituitary preparation was therefore repeated, under conditions strictly analogous to those used for prolان. Excess reagent was not separated after reaction and the assay was performed with the addition of zinc as ZnSO_4 to increase sensitivity.

20.0 mg. of the pituitary preparation were dissolved in 5 cc. of water containing 200 mg. of NaHCO_3 . One aliquot was kept as a control. To the other were added 55 mg. of dimethyl sulfate and 100 mg. of NaHCO_3 . After 1 hour at room temperature, 10.0 mg. of Zn (as ZnSO_4) and acetic acid (to take the pH to 5.0) were added to each aliquot. The final volumes were 10 cc. The ovaries of the rats dosed with the control solution averaged 85 mg. in weight; those of the rats given the methylated preparation, 14 mg. One-half dose of the control solution produced 35 mg. ovaries.

Results

In mildly alkaline solution prolان was more than 90 per cent inactivated by acetylation, benzylation, and by reaction with naphthoquinone sulfonate or H_2O_2 . The pituitary gonadotropic preparation responded in a strictly analogous manner. In acid solution little loss of activity was noted with acetic anhydride, and only partial inactivation with naphthoquinonesulfonic acid, or with H_2O_2 .

In mildly alkaline solution, phenyl isocyanate and CS_2 likewise produced considerable inactivation. Here again the response roughly parallels that for the pituitary gonadotropic preparation.

Prolان was stable to moderate excess of nitrous acid, formaldehyde in both alkaline and acid solution, iodoacetate, and acetaldehyde. (Prolان is destroyed by a high concentration of formaldehyde.)

Stability of prolان to iodine was governed by the pH. At pH

3.5 no loss of activity was noted. At pH 8.5, it was more than 90 per cent inactivated.

5 per cent of the activity of prolan was recovered after solution in concentrated H_2SO_4 . In our studies on the pituitary gonadotropic preparation, no activity was reported after solution in concentrated H_2SO_4 . In the latter studies, the method of assay was not delicate enough to distinguish between lack of any activity and 5 per cent recovery.

A high degree of inactivation was produced by 0.1 N HCl in as short a time as 30 minutes at 40° , and in 24 hours at room temperature. The experiments with 0.1 N HCl, with H_2O_2 , and with diazobenzenesulfonic acid are the only ones in the series in which there is a significant discrepancy in the values obtained by the different methods of assay. In the coupling experiment there is a suggestion of the survival of a follicle-stimulating fraction. In the experiment with 0.1 N HCl there is likewise a suggestion of the survival of the follicle-stimulating fraction. In this case, however, the assay for seminal vesicle increases parallels the result for ovarian weight increases, while the prostate gland weights parallel the assay by presence of corpora lutea and there is therefore no evidence for a change in ratio of gametotropic to interstitial cell-stimulating hormone. A lessened response to prostate hypertrophy as compared with ovarian and seminal vesicle hypertrophy is noted in the experiments with 0.1 N HCl and with H_2O_2 .

Prolan was relatively stable to dimethyl sulfate in mildly alkaline solution in contrast to the pituitary preparation, which was inactivated under strictly analogous conditions.

DISCUSSION

The results of the foregoing experiments indicate a marked similarity in the stability of prolan and the pituitary sex hormone to a wide range of chemical reactions. In the twenty-two reactions and conditions studied, it is only possible to pick out one in which prolan shows sensitivity to a condition under which the pituitary preparation is stable or stability to a condition under which the latter preparation is sensitive (assay by ovarian weight increases). The characteristic reagent is dimethyl sulfate in mildly alkaline solution.

As in the case of the numerous studies of insulin, no structural groups or nuclei can be characterized as responsible for the physiological activity. Certain characteristic trends, nevertheless, are revealed: (1) the relative stability to dimethyl sulfate, formaldehyde, and nitrous acid in contrast to the extreme sensitivity to acylation and reaction with naphthoquinone sulfonate; (2) the sensitivity to mineral acid, with a sharp boundary line of stability (pH 3.0); (3) a more pronounced tendency to be inactivated by reagents in mildly alkaline solution (acylation, H_2O_2 , I, naphthoquinone sulfonate) than in mildly acid solution.

While the four methods of assay do not in all cases give identical results, a clear cut separation of two hormones is never indicated.

SUMMARY

1. Prolan in mildly alkaline solution is more than 90 per cent inactivated by acetylation, benzylation, reaction with β -naphthoquinone sulfonate, or reaction with H_2O_2 ; the activity is considerably reduced by reaction with phenyl isocyanate, CS_2 , and diazobenzene sulfonate.

2. Prolan is stable to moderate excess of nitrous acid, formaldehyde in both alkaline and acid solution, iodoacetate, and acetaldehyde.

3. Prolan is stable to iodine at pH 3.5 and is mostly destroyed at pH 8.5.

4. Prolan is unstable at pH 2.0.

5. In contrast to the pituitary preparation, prolan is relatively stable to dimethyl sulfate in mildly alkaline solution.

6. In the twenty-two reactions studied, no clear cut separation of two hormones was revealed. Hypertrophy of the prostate gland as compared with that of ovaries and seminal vesicles was lessened in three instances. Follicle stimulation was increased in one instance.

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CHEMICAL STUDIES ON THE ADRENAL CORTEX

III. ISOLATION OF TWO NEW PHYSIOLOGICALLY INACTIVE COMPOUNDS*

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In Paper II of this series (1) we described the isolation from adrenal extracts of four crystalline, nitrogen-free compounds, which were found to be physiologically inactive when tested on the adrenalectomized dog. Three of these compounds, besides others not encountered by us, have meanwhile been obtained independently by Reichstein (2);¹ the fourth is apparently identical with the substance designated as the adrenal cortical hormone by Kendall, Mason, McKenzie, Myers, and Koelsche (3). Analytical data obtained on these compounds in the various laboratories indicated the presence of 21 carbon atoms, and 5 oxygen atoms with varying functions. Reichstein subsequently showed that at least three of the compounds of this series (his Compounds A, C, and D) could be degraded to the same diketone² $C_{19}H_{28-28}O_5$, which possesses strong male sex hormone action and is in all probability closely related to androstanedione (4). This highly important observation, besides revealing a chemical relationship which might be concerned in the clinical syndrome of adrenal virilism, can be taken as strong presumptive evidence that the afore-

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¹ The identity of our Compound A with Reichstein's Compound A, and of our Compound D with his Compound C has been established by mixed melting point determinations with samples kindly sent us by Dr. Reichstein.

² We had also obtained this ketone by chromic acid oxidation of Compound A some time before the publication of Reichstein's results.

mentioned C_{21} compounds are derivatives of pregnane, or more probably of allopregnane.

The present communication deals with the isolation of a new member of this series, of the composition $C_{21}H_{28}O_6$, which is provisionally designated Compound F. This substance constitutes the bulk of the "chloroform-insoluble crystals" (Fractions 10 and 11, Diagram 2, Paper I in this series (5)) present in the final active fractions. We had in hand a few mg. of this compound in almost pure form about a year ago. A preliminary description including melting point, analysis, and bioassay was included in our previous paper ((1) p. 606). However, its designation as a new chemical individual was deferred pending the isolation of larger amounts and a more detailed study of its properties. At that time only the analytical data set it apart from Compound B, with which it appeared similar in several other respects. We later found that this compound can be easily distinguished from all other compounds previously isolated by us by the fact that it absorbs light in the ultraviolet region. Furthermore, the intense green fluorescence of its solution in concentrated sulfuric acid affords a simple means of differentiating Compound F from related compounds. Its ultraviolet absorption curve shows a strong single maximum at $240\text{ m}\mu$ (Fig. 1, Curve A). This type of spectrum is characteristic for α,β unsaturated ketones. The ketonic character of the compound was ascertained by the preparation of a disemicarbazone. The ultraviolet absorption curve of the latter shows a strong maximum at $270\text{ m}\mu$, $\epsilon = 29,000$, and a second indistinct peak at about $240\text{ m}\mu$, $\epsilon = 19,000$ (Fig. 1, Curve D). The higher peak is almost identical with the single peak spectrum of the semicarbazone of cholestenone ((6), $273\text{ m}\mu$, $\epsilon = 26,300$), adducing additional evidence that Compound F is an α,β unsaturated ketone. A disemicarbazone $C_{23}H_{36}O_6N_6 \pm H_2$ of identical physical properties has been obtained by Reichstein directly from physiologically active fractions by treatment with semicarbazide (his Compound F).³

On treatment with *p*-nitrobenzoyl chloride in pyridine, Com-

³ On treatment of our disemicarbazone with concentrated sulfuric acid the green fluorescence appears very slowly. Dr. Reichstein informs us that his Compound F behaves in the same manner. He has meanwhile also obtained the free diketone (private communication).

pound F yielded a crystalline derivative which after two recrystallizations melted at 220–221° (corrected). Analysis indicated that this product was a mononitrobenzoate, contaminated with some dinitrobenzoate.

Further bioassays on Compound F confirmed the preliminary negative result reported last year ((1) Table I, Preparation 11). The compound has since been assayed on three more adrenalect-

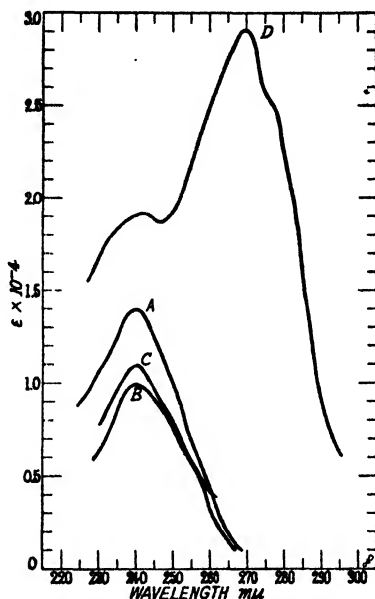


FIG. 1. On the abscissæ are given readings for wave-lengths in $m\mu$; on the ordinates, molecular extinction coefficient $\epsilon = (1/c \cdot l) \log_{10} (I_0/I)$ (nomenclature of International Critical Tables). Curve A, Compound F; Curves B and C, active fraction after separation of Compound F (two different preparations); Curve D, disemicarbazone of Compound F. Solvent, alcohol.

tomized dogs and failed to maintain them on dosage levels of 50, 50, and 100 micrograms per kilo of body weight per day. The test animals came promptly into typical acute insufficiency in 5, 5, and 6 days, respectively. The usual changes were observed in the serum sodium and blood non-protein nitrogen levels; namely, a diminution in the former and a marked elevation in the latter.

Before the publication of Reichstein's paper in which almost

compelling evidence for the pregnane structure of the $C_{21}H_{30}O_6$ compounds was adduced, we suspected a relationship of our Compound F with the α, β unsaturated diketone progesterone. Accordingly two batches of Compound F were sent to Dr. W. M. Allen of the University of Rochester, who kindly assayed the compound for corpus luteum activity.⁴ The result was entirely negative. No progestational modification of the rabbit endometrium was obtained with as high a dose as 15 mg. per test animal. This is not surprising in view of the high specificity of progesterone action, for which only a few exceptions (7) are known.

The isolation of Compound F was facilitated by the use of Girard's reagent, betaine-hydrazide hydrochloride (8), which had been employed by Reichstein to separate the more reactive ketones present in adrenal extracts. As we have pointed out on previous occasions (1,9), "ether concentrates" react very readily in the cold with phenylhydrazine derivatives with the formation of bulky amorphous precipitates, which contain all of the physiologically active material. They consist of mixtures of hydrazones of the hormone and of other, physiologically inert, ketones. Since Compound F is precipitated instantaneously by phenylhydrazine under these conditions, it must be present, probably as the principal contaminant of the hormone, in these mixtures. If the active material remaining after removal of the fractions containing the other crystalline compounds be treated with Girard's reagent and separated into "ketonic"⁵ and "non-ketonic" fractions, the hormone and Compound F are for the most part retained in the "ketonic" fraction. A second treatment of the "non-ketonic" part with Girard's reagent is necessary to make the separation more complete.

It should be pointed out here that only the more reactive, probably for the most part α, β unsaturated, ketones are fixed by Girard's reagent under the conditions employed. The "non-ketonic" fraction still contains ketones which do not combine readily with hydrazine derivatives. Thus Compound B, which possess a

⁴ We wish to extend our sincere thanks to Dr. Allen for his kind cooperation.

⁵ The terms "ketonic" and "non-ketonic" are used in an arbitrary manner to designate the two fractions obtained with Girard's reagent under the conditions employed by us.

reducing carbonyl group, but does not form an insoluble phenylhydrazone, is always encountered in the "non-ketonic" fraction, if the fractionation with Girard's reagent is instituted at an earlier stage of the fractionation scheme. These results obtained by means of Girard's reagent, particularly those pertaining to the distribution of physiological activity, are in accord with the data published by Reichstein, who employed the adrenalectomized rat muscle test of Everse and de Fremery (10) for assaying his fractions.

The syrup remaining after removal of as much of Compound F as can be separated by crystallization from the "ketonic" portion contains the physiological activity. This material shows strong specific absorption in precisely the same location as Compound F, namely at $240\text{ m}\mu$, but of somewhat lower intensity (Fig. 1, Curves B and C). Undoubtedly some of Compound F is still present in the active syrup and must account for a part of this absorption. Amorphous semicarbazones, obviously mixtures, which were obtained from such an active preparation showed a nitrogen content of 13.6 per cent, while the disemicarbazone of Compound F contains 17.8 per cent. On the assumption that the average molecular weight of the ketones present in the syrup is of the same order of magnitude as that of Compound F,⁶ the nitrogen figure speaks for a mixture of di- and monoketones, with a preponderance of the latter (54 per cent). This figure is probably too low on account of the greater solubility of monosemicarbazones. Even if all of the diketonic portion were represented by Compound F, which is unlikely, it could not be responsible for all of the light absorption at $240\text{ m}\mu$. Consequently, at least a part of the monoketonic fraction also consisted of α,β unsaturated ketones. Further fractionation of the syrup itself, or of the mixture of semicarbazones, has not led to the separation of crystalline compounds. For the present we may tentatively assume that the hormone itself is an α,β unsaturated ketone, probably related to Compound F. The presence of an α,β unsaturated keto group would explain the well known instability of the cortical hormone towards alkali, which it shares with progesterone and testosterone. However, it must be kept in mind that a reducing

⁶ Reichstein's analysis of an amorphous physiologically active preparation of similar description and derivation indicated a composition $\text{C}_{22}\text{H}_{30}\text{O}_4$.

carbonyl group (in the side chain, see discussion below) would also confer alkali instability on the hormone molecule. Reichstein has proved conclusively that in at least two of the reducing compounds of the C_{21} series the reducing group is located in a two-membered side chain which can be removed by oxidation with chromic acid.

A preliminary account of these findings was given in March, 1936 before the American Society of Biological Chemists (11). At the same meeting Kendall, Mason, Myers, and Allers (12) reported the isolation from adrenal extracts of a compound $C_{21}H_{30}O_5$ (Compound E of their series) in which the oxygen atoms were at that time considered to be present in the form of three hydroxyl groups, an aldehyde group, and an ether linkage.⁷ However, in a more recent publication (14) they revise the formula to $C_{21}H_{28}O_5$ and state that two carbonyl groups and three hydroxyls are present. The absorption spectrum corresponds to that of our α,β unsaturated ketone, and it appears probable that the compound is identical⁸ with our Compound F. On oxidation with periodic acid their Compound E yielded an acid $C_{20}H_{26}O_5$, and with chromic acid an α,β unsaturated diketone $C_{19}H_{24}O_3$, which is reported to possess male hormone activity. The structural formulas devised by the Mayo Clinic workers for their Compound E and the androgenic diketone derived from it are entirely analogous to the formulas first proposed by Reichstein for his saturated Compounds A, C, and D, and the androgenic diketone obtained from these with chromic acid. Kendall and coworkers accordingly visualize their Compound E as a 3,20-diketo-17, 21-dihydroxy- $\Delta_{4,6}$ pregnanediene, with an additional, probably tertiary, hydroxyl group in still unknown location. While the principal

⁷ A more detailed description of this compound has meanwhile been published by the Mayo Clinic workers in the July issue of this *Journal* (13). Its composition is still given there as $C_{21}H_{30}O_5$, and the preparation of a mono-2,4-dinitrophenylhydrazone is described. The remaining oxygen atoms are not characterized in this publication.

⁸ Soon after the Washington meeting Dr. Kendall kindly furnished us with a sample of his Compound E, then still designated $C_{21}H_{30}O_5$. There was no depression of its melting point ($201-208^\circ$) when it was mixed with a sample of our Compound F, melting at $202-210^\circ$. This result was communicated to Dr. Kendall on April 4, but was not mentioned in the May, 1936, article (14).

features of this formula are probably correct on the strength of their degradation work, and of Reichstein's on the related saturated compounds, we cannot agree with the proposed allocation of the second double bond (the existence of which is as yet deduced only from the empirical formula) to the 6-7 position in the pregnane skeleton. The evidence given for placing this double bond in conjugation to the 4-5 double bond, namely the weakness of the coloration obtained with tetranitromethane, is in our opinion inadequate, since it is known that α,β unsaturated ketones do not respond readily to this reagent (15). The same is probably true of single ethylenic bonds between carbon atoms shared by two adjoining rings. Moreover, the spectroscopic results argue against a doubly conjugated bond system, such as is provided in Kendall's formula, which should exhibit a spectrum more complex, and extending into higher wave-lengths than the single peak type given by Compound F. For instance, the absorption curve of 7-keto- $\Delta_{5,6}$ cholestadiene shows a maximum at $280 m\mu$ and end-absorption below $240 m\mu$, with a minimum at $250 m\mu$ (16). The location of the conjugated ethylenic ketone system in Ring B, rather than in Ring A, cannot explain the shift of the maximum into a higher wave-length range. The acetate of 7-oxocholesterol (3-hydroxy-7-keto- Δ_5 cholestene) exhibits the same typical $240 m\mu$ band as cholestenone (unpublished work of Miss S. Ratner of this laboratory).

As pointed out above, Compound F when dissolved in concentrated sulfuric acid gives rise to an orange-red solution with extremely intense green fluorescence, a reaction not given by the other members of the $C_{21}H_{36}O_6$ series. Among all the compounds related to sterols or bile acids which have been tested (including cholestenone, progesterone, and testosterone) only apocholic acid gives the reaction with equal intensity but much more slowly. The single ethylenic bond in apocholic acid is situated in the 8-9, or 8-13 position, the two hydroxyl groups in positions 3 and 12. The possibility that the second double bond in Compound F is located between carbon atoms shared by two rings is under investigation.

Kendall, Mason, and Myers (14) report that their Compound E shows definite physiological activity when tested on the adrenalectomized rat by Ingle's method. Besides maintaining the life and

normal working capacity of the test animal, it is said to prevent the rise in blood urea characteristic of incipient insufficiency. However, "the quantitative activity of Compound E is much less than that of cortin itself."⁹ No dosage figures are given, so that a comparison with the figures available in Reichstein's paper (2), which were secured by a somewhat similar method of bioassay, cannot be made. Concerning our own negative results on the adrenalectomized dog, we concede the possibility that Compound F may be effective in larger doses than those employed by us, but if this be the case, it follows from our data that its efficiency must be considerably less than one-fortieth of that of our active fractions.

Compound G—While Compound F was isolated from the final physiologically active fractions, another new compound, designated Compound G, was recovered from a comparatively inactive fraction set aside early in the isolation process; namely, the material remaining in the ether phase after extraction of the hormone with dilute hydrochloric acid (Diagram 1, Paper I (5)). A fraction of this type, derived from 500 kilos of glands, was subjected to treatment with pentane and acetone in the manner described by Reichstein (2). Glycerol monopalmitate could, in accordance with the experience of this author, be separated at this point. When the resulting pentane solution was extracted with 10 per cent methanol, a yellowish, flocculent product accumulated at the interphase, which remained insoluble throughout the subsequent washings with dilute methanol. From this material small amounts of a new compound (G), of the probable composition $C_{21}H_{34}O_3$, were isolated by fractional crystallization from alcohol. 1 of the oxygen atoms is present as a keto group, since the compound forms a crystalline monosemicarbazone. The analyses of the semicarbazone are too low in carbon for a derivative of $C_{21}H_{34}O_3$ and would fit better for derivatives of C_{20} or C_{19} compounds, but the data obtained on the original compound are probably more reliable. In view of the evidence cited above for the presence of a

⁹ It is not clear from this statement whether by "cortin" is meant the crystalline compound $C_{20}H_{30}O_3$ or $C_{21}H_{34}O_3$, which the authors considered to be the life maintenance hormone in 1934 (3), and the physiological importance of which was reaffirmed in 1935 (17), or whether this term refers to active amorphous fractions of the type we have described in our papers.

pregnane skeleton in the compounds of the $C_{21}H_{32}O_6$ series, it is not unlikely that Compound G will turn out to be related to one of the isomeric pregnanolones.

From the solubility properties and mode of isolation of Compound G, cortical hormone activity could hardly be expected. In a preliminary assay in one adrenalectomized dog Compound G was found to be physiologically inactive at a dosage level of 50 micrograms per kilo of body weight per day. The test animal developed typical acute insufficiency in 5 days.

EXPERIMENTAL

Compound F—As has been mentioned in the theoretical part, Compound F can be obtained directly by fractional crystallization with alcohol from the "chloroform-insoluble crystals" (Fractions 10 and 11, Diagram 2 (5)). However, in these fractions it occurs as a mixture with at least one other crystalline compound, presumably Compound D, from which it can be separated only with difficulty. Fractionation with Girard's reagent yields more tractable products. This treatment can be interposed either after separation, in the manner described previously, of Compound B, or more conveniently before this step. We have encountered difficulties in obtaining significant amounts of this compound (B) in working up 100 kilo batches of glands, and find it more advantageous now to isolate it from the "non-ketonic" fraction of the Girard process. In order to recover Compounds A and C we also have returned now to the ether distribution procedure in preference to the alternative benzene method. In the following we describe the procedure now in use as applied to an ether concentrate derived from 500 kilos of glands. The benzene residue (Fraction 1) and benzene deposit (Fraction 2) obtained from 6.8 gm. of ether concentrate were worked up in the usual manner for Compounds A and C and the hormone recovered from the aqueous mother liquor by washing with benzene. This benzene solution was combined with the petroleum ether precipitate derived from Fraction 3, after separating off Compound E (leucylproline anhydride). The combined material, weighing 3.4 gm., was dissolved in a solution containing 3.4 gm. of Girard's reagent in 2.7 cc. of water and 24.5 cc. of ethyl alcohol. This solution was allowed to stand 4 hours at room temperature, and after dilution with water

to 70 cc. extracted five times with 70 cc. of chloroform, which had been previously shaken with 35 per cent alcohol. The combined chloroform solutions contained the "non-ketonic" material, but also small amounts of Compound F and of the hormone, which can be recovered by a second treatment with Girard's reagent. The ketones fixed by the hydrazide in the dilute alcohol phase were then liberated by the addition of 0.7 cc. of concentrated hydrochloric acid, and after standing overnight extracted with chloroform in the same manner as the non-ketonic portion. Back washing of the chloroform extracts with water was avoided, since considerable amounts of both "ketonic" and "non-ketonic" material pass into the aqueous phase. It was, however, ascertained by blank experiments that contamination of the chloroform extracts with the reagent or with acid is negligible.

The solution containing the "ketonic" fraction was brought to dryness *in vacuo*. The residue consisted of a yellow syrup weighing 1.18 gm. It was taken up in 4 cc. of dry chloroform and the solvent allowed to evaporate slowly in a desiccator over paraffin and CaCl_2 . Small amounts of whetstone-shaped crystals slowly form in the solution. After all of the solvent had disappeared, the syrup was again taken up in 2 cc. of chloroform in which it dissolved slowly, while the crystals remained undissolved. The solution was again brought to the syrupy stage in the same manner, when it deposited more crystalline material. After five more treatments with fresh solvent the amount of crystalline material did not seem to increase any more. The crystals were then transferred gradually to a fritted glass filter with small portions of chloroform and filtered by suction. If too much chloroform is used at once to dissolve the syrup, a yellow flocculent precipitate forms which contaminates the crystals. This was not observed in earlier experiments in which the Girard process was employed in a later stage of the fractionation scheme. The crystals on the filter, together with some yellowish white, partly crystalline material remaining in the original vessel, weighed 243 mg. The whole amount was taken up in about 5 cc. of warm acetone. An undissolved, amorphous, yellow product was centrifuged and washed with acetone. The acetone solutions were brought to dryness and taken up in 0.7 cc. of absolute alcohol. On prolonged standing in the ice box 60 mg. of the crude compound were obtained. Re-

peated recrystallization from 95 per cent alcohol yielded 30 mg. of analytically pure substance. Only small additional amounts could be recovered from the original mother liquor. Other measures to effect better recovery failing, the final residue was again treated with chloroform, which seems to be the only solvent promoting crystallization in the presence of the syrupy by-products. However, chloroform cannot be used advantageously for recrystallization, since the solubility of the crystals in this solvent is low and the temperature coefficient unfavorable. The disappointingly low yield of the pure product finally obtained from the original chloroform-insoluble, mainly crystalline material is probably not due to decomposition, for the compound when once purified beyond a certain stage seems perfectly stable in hot solvents and easily crystallizable. It is more likely that the difficulties encountered in recrystallizing the cruder products are caused by contaminations profoundly influencing the solubility.

- Compound F crystallizes from alcohol in the form of rhombohedral platelets. In the form of small crystals or a fine powder it melts with decomposition at 203–209° (corrected) in the open capillary. Single large crystals show higher melting points up to 217°, undoubtedly because decomposition is then delayed. The compound is fairly soluble in cold methyl and ethyl alcohols and in acetone, but is much less so in ether, benzene, chloroform, and only slightly soluble in water. Its specific rotation $[\alpha]_D^{25}$ is $+209^\circ \pm 1^\circ$ (1.2 per cent solution in 95 per cent alcohol). Kendall and collaborators report an $[\alpha]_{5461}^{25}$ of $+248^\circ$ in the same solvent.

The compound was dried for analysis in a high vacuum (10^{-3} mm. of Hg) at 80°.

<i>Analysis</i> — $C_{11}H_{20}O_6$.	Calculated.	C 69.96, H 7.83
	Found.	" 69.75, " 7.77
		" 69.71, " 7.87
		" 69.84, " 7.89 (previously reported)

Compound F gives negative Liebermann-Burchard, Pettenkofer, and Rosenheim reactions. The fluorescence reaction with concentrated sulfuric acid has been mentioned before. The reaction is carried out best on a few crystals crushed between a microscope slide and a cover-glass. The pigment formed is not soluble in chloroform. Compound F does not precipitate with digitonin from a 0.1 per cent solution in 80 per cent ethyl alcohol. It re-

duces Benedict's solution on heating. The fuchsin-sulfurous acid reaction is negative.

Disemicarbazone—11.7 mg. of Compound F dissolved in 90 per cent alcohol were heated with an excess of semicarbazide-acetate for 2 hours at 70°, and then allowed to stand 24 hours at room temperature. The granular precipitate obtained on partial evaporation of the solvent and dilution with water was washed several times with water and dried (15 mg.). The product was dissolved in 6 cc. of hot alcohol and the volume quickly reduced by boiling to 2 cc. A semicrystalline powder deposited on slow cooling. The compound gradually decomposes on heating above 250° and is not completely liquefied at 300°. The product was dried for analysis at 110° and 2 mm. of Hg.

<i>Analysis</i> — $C_{23}H_{34}O_5N_6$.	Calculated.	C 58.19, H 7.22, N 17.72
	Found.	" 57.34, " 7.14, " 17.1, 17.8

p-Nitrobenzoate—12.4 mg. of Compound F and 30 mg. of *p*-nitrobenzoyl chloride were dissolved in pyridine and the solution kept at 100° for 2 hours. The crude reaction product obtained by precipitation and washing with ice water weighed 9.5 mg. It was recrystallized twice from alcohol, giving rosettes of prisms, melting at 220–221° (corrected) with decomposition. Although the melting point was fairly sharp and the appearance of the crystals homogeneous, the analysis indicated that the product was a mixture of a mono- and a di-*p*-nitrobenzoate. The carbon and hydrogen figures are somewhat uncertain, since only 0.748 mg. was used for the analysis.

<i>Analysis</i> — $C_{28}H_{31}O_5N$.	Calculated.	C 65.98, H 6.14, N 2.75
$C_{35}H_{34}O_{11}N_2$.		" 63.81, " 5.21, " 4.26
	Found.	" 64.9, " 6.1, " 3.24

The reducing action towards Benedict's solution shown by Compound F is abolished with the disemicarbazone, as would be expected, but not with the nitrobenzoylation product. The latter also gives the green fluorescence with sulfuric acid instantaneously, while the same reaction develops only very slowly with the disemicarbazone.

Compound G—The ether-soluble, acid-insoluble side fraction obtained in the first fractionation step applied to permutit-purified

material (Diagram 1 (5)) was extracted thoroughly with pentane. 89 gm. of material of this type were worked up. From the pentane-soluble part 4 gm. of crude glycerol monopalmitate crystallized on chilling. The mother liquor was added to the tarry residue of the pentane extraction and the whole fraction distributed between 500 cc. of pentane and 100 cc. of 20 per cent methanol. A yellow flocculent precipitate and some dark colored tar remained undissolved. The former accumulated at the interphase and did not go into solution on subsequent distributions with fresh portions of 20 per cent methanol. The further treatment of the methanol phase, aiming at the recovery of some cortical hormone, and of adrenosterone, which should both be contained in this fraction according to Reichstein (18), will not be described here. The yellow flocculent precipitate was collected, dried, and extracted repeatedly with small portions of pentane, which removed some pigment present. 154 mg. of still pigmented material were recovered. This was crystallized twice from alcohol and yielded 45 mg. of irregular platelets melting at 237–251°. The mother liquors contained another substance crystallizing in needles; this was not investigated further. Two more recrystallizations of the top fraction yielded an apparently homogeneous product, which consisted of large platelets resembling distorted hexagons, softened at 254°, and melted with some decomposition at 264° (corrected). On further recrystallization the melting point remained constant, but did not become sharper. The compound is less soluble in alcohol than the previously isolated compounds with the exception of Compound D. It is only sparingly soluble in hot acetone, and insoluble in water. Its specific rotation $[\alpha]_D^{25}$ is $+38^\circ \pm 1^\circ$ (0.64 per cent in 95 per cent alcohol). It does not reduce hot Benedict's solution. For the bioassay only a less pure product (m.p. 253°), recovered from the mother liquors, was available. On account of its insolubility in water it was injected in suspension.

Analysis— $C_{21}H_{32}O_3$ (332.3). Calculated. C 75.85, H 9.71

$C_{21}H_{34}O_3$ (334.3). " 75.39, " 10.25

Found. " 75.13, " 10.22

(After 1 more recrystallization)

" 75.73, " 10.14, mol. wt.
(camphor) 334

Semicarbazone—The semicarbazone was prepared from 11.6 mg. of Compound G in the usual manner. It deposited in large oblong

plates directly from the hot alcoholic solution. The product was recrystallized from a larger volume of absolute alcohol; yield 10.2 mg. The semicarbazone melts at 263–265° (corrected) with gas evolution. It is even less soluble in hot alcohol than the disemicarbazone of Compound F.

Analysis— $C_{22}H_{37}O_4N_3$. Calculated. C 67.46, H 9.53, N 10.74
Found. " 66.40, " 9.35, " 11.02

Glycerol Monopalmitate—The crude preparation was recrystallized five times from benzene and acetone and then melted at 71° (corrected).

Analysis— $C_{19}H_{38}O_4$. Calculated. C 69.03, H 11.60
Found. " 69.25, " 11.43

We wish to thank Mr. William Saschek of this Department for the microanalyses reported in this series of papers.

SUMMARY

The isolation and properties of two new physiologically inactive compounds from adrenal extracts are described.

The first compound is an α,β unsaturated diketone of the formula $C_{21}H_{28}O_5$ and is probably closely related to several of the inactive compounds isolated previously. The amorphous active fractions remaining after separation of this compound are mixtures of ketones, for the most part also α,β unsaturated.

The second compound is a monoketone of the probable composition $C_{21}H_{34}O_3$.

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A STACK OF CONSTANT VOLUME FOR RESPIRATION EXPERIMENTS WITH HUMANS

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In any study of the respiratory exchange of humans by the open circuit principle a method for collecting the expired air, either in its entirety or in proportional part, is necessary. If the total volume of expired air is collected, as is the case when the spirometer or the Douglas bag method is employed, it is possible at the end of the respiration experiment to obtain a sample of the thoroughly mixed gas for analysis. If the expired air is not collected completely but its volume is measured as it is being discharged (either by a wet gas meter, as in the Zuntz-Geppert method, or by two dry gas meters in series, as in some of the Nutrition Laboratory's forms of respiration apparatus), a perfect aliquoting system is necessary, to collect from the ventilating air current a sample of expired air simultaneously with and proportional to the total volume being metered. These procedures are all based upon an accurate knowledge of the total volume of exhaled air. This means that the initial reading of the spirometer level or of the gas meter should be precise, and that the final reading should be equally as accurate. Strictly speaking, these readings should be checked by a second observer. A method of studying the respiratory exchange of humans is proposed that eliminates the necessity for measurement of the volume of expired air, inasmuch as the expired air is delivered into a recipient of known and constant volume. The basic principle of this method rests on the fact that expired air has a density greater than that of outdoor air. The procedure is to conduct the expired air (as it comes from the lungs of the subject, who is breathing outdoor air) into the bottom of a tall cylindrical pipe or stack previously filled with outdoor air.

The expired air remains in the bottom of the stack and gradually expels the outdoor air through an exit at the top. The entire volume of air discharged from the lungs during a given period of time can thus be collected without significant loss of expired air from the top of the stack. At the end of the collection period (about 10 minutes) the air in the apparatus is thoroughly mixed, and a sample is withdrawn for analysis of its carbon dioxide and oxygen content. The results of such analyses, when applied to the known volume of the apparatus, indicate the carbon dioxide production and the oxygen consumption of the subject during the exact time of collection.

The density of the air exhaled from the lungs is affected by its carbon dioxide content, its water vapor content, and its temperature. Expired air contains about 3.5 per cent carbon dioxide, which makes it heavier than ordinary room air, but it is saturated with water vapor which, *per se*, makes it lighter than room air, and it leaves the lungs at a temperature of not far from 30°, which makes it lighter than room air at laboratory temperature. The expired air, by virtue of its temperature (30°), would tend to rise when discharged into a stack holding outdoor air (0.030 per cent carbon dioxide and 20.940 per cent oxygen) saturated at about 22°. This tendency to rise would prevent or disturb a stratification based on density. By cooling the air coming from the lungs to a temperature a degree or so below that of the air in the stack, this tendency of the expired air to rise in the stack is prevented. The percentage of carbon dioxide (about 4 per cent) and the slightly cooler temperature of the expired air both make for a gas denser than outdoor air. Hence the expired air will remain in the base of such a tall pipe or stack and minimize interchange with the outdoor air by convection currents caused by difference in temperature. Because the expired air enters the stack through a small pipe and is discharged into a large volume and because the interior of the stack is provided with baffle plates, the motion of the expired air is greatly slowed as it enters the stack. Under these conditions the main, if not the sole, cause for mixture of the two gases will be simple diffusion. It is this latter factor that determines the shape of the stack, for obviously the taller the stack the longer the period of time before any portion of the expired air can escape at the top of the stack owing to diffusion.

In basal metabolism measurements the experimental periods are usually 10 minutes long, although in many laboratories, especially where the modern form of spirometer apparatus is employed, the periods are commonly 6 minutes in duration. The larger the ventilation of the lungs, that is, the greater the discharge of expired air in 10 minutes, the greater will be the possibility for loss of respiratory carbon dioxide from the top of the stack. On the assumption that a human subject might exhale the rather considerable volume of 7.5 liters of air per minute or 75 liters per 10 minutes, on the assumption that there would be a reasonable degree of stratification of the expired air in the stack; and with the use of a simple system of baffle plates, which minimize convection currents and diffusion, the stack was designed to have a volume of approximately 120 liters.

Construction of Stack—The stack must be absolutely air-tight and, as the air in it will be, for the most part, saturated with water vapor, it is preferable that the material used in its construction should be rust-resisting. Sheet copper is rustless, but in the interests of economy galvanized sheet iron, No. 26 gage (0.48 mm. thick), was employed in the commonly available form of sections of so called 10 inch "furnace smoke pipe." These sections of pipe are approximately 25 cm. in internal diameter and 60 cm. in length. One end of each section tapers slightly, so that it is easily telescoped into the non-tapering end of another section and a reasonably tight, but not air-tight, joint obtained. Four of these sections were joined together, so that the total length of the stack was about 2.3 meters. Where there was a seemingly large crack or poor closure, a bit of physicist's wax was put in place. Then a strip of adhesive tape 5 cm. wide was bound around each horizontal seam (see *h, h*, Fig. 1), and the closure thus formed was given a coat of shellac. This method of closure, which was suggested by Mr. Robert C. Lee of the Nutrition Laboratory based upon his clever method of closing the door of the large respiration chamber for elephants (1), has been most satisfactory. This particular form of connection was obviously desirable during the development of the apparatus when the stack was frequently dismounted, but now that the accuracy of functioning of the apparatus has been proved, it would be satisfactory to solder the joints of the four sections permanently. The two ends of the pipe formed by

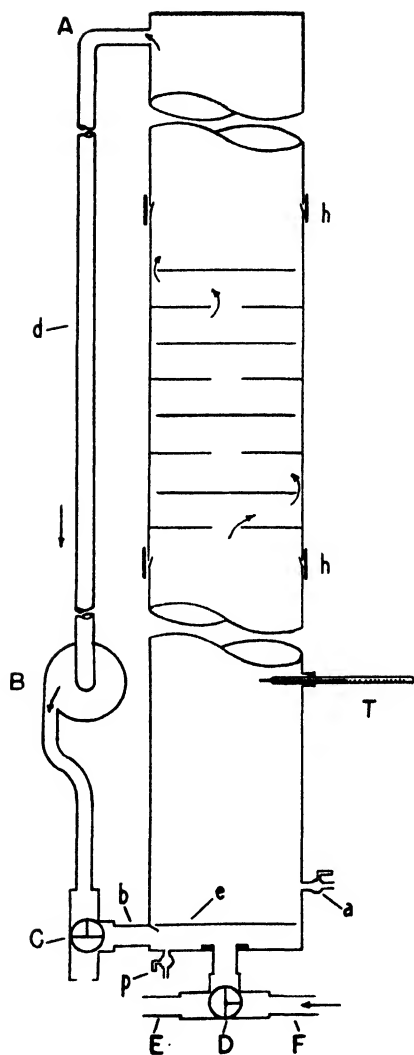


FIG. 1. A stack respiration apparatus of constant volume for respiration experiments with humans. The stack (not shown in its entire length in the diagram) is in four sections, telescoped together and made air-tight with wax and adhesive tape, *h, h*. *A*, elbow through which air leaves the stack; *d*, pipe leading to rotary air impeller, *B*; *C* and *D*, 3-way valves; *E* and *F*, pipes for conduct of air; *a*, pet-cock for withdrawal of air sample from stack; *b*, nipple connecting valve *C* to base of stack; *e*, baffle plate; *p*, pet-cock for removal of water in base of stack; *T*, thermometer. Arrows indicate direction of air flow.

the joining of these four sections are closed with caps or disks of galvanized sheet iron, well soldered in place. The seams lengthwise of the pipe are likewise reinforced by solder. In this way the stack has been made absolutely air-tight and will stand a pressure of several cm. of water for some time. Thus absence of leakage of air out of or into the stack is insured.

Baffle Plates—In the third section from the bottom of the stack are soldered a series of eight baffle plates. The lowest of these is soldered to the inside of the pipe and has in its center a hole 5.5 cm. in diameter. The second of the series of plates is held about 3 cm. above the first plate by three small feet, which are soldered onto the first plate. This particular plate is smaller in diameter than the first plate, so that there is an annular space between its outside circumference and the inner wall of the stack of approximately 8 mm., to allow free passage upwards of the gas. This arrangement of two baffle plates is repeated four times, the top plate being the one that deflects the air against the inside wall of the pipe. In our experimenting with the rate of diffusion, three fine mesh, wire netting screens of copper wire were soldered to the inner wall of the top section of the pipe near its base. It is not certain that all the eight baffle plates and three screens are imperative. They were added in the course of our experimenting; the expense is slight and the ultimate result is successful.

Connections at Top and Base of Stack—At the top of the uppermost section of the stack a $\frac{3}{4}$ inch elbow, *A*, is soldered to the side. In the center of the base of the stack is an opening, to which a $\frac{3}{4}$ inch, 3-way valve, *D*, can be either soldered or rigidly screwed by use of a close nipple and a lock nut with rubber gasket either side of the base. This nipple and lock nut project inside the base of the stack approximately 1 cm. In addition, in the side of the lowermost section at the base the nipple, *b*, is soldered, to which is screwed a second $\frac{3}{4}$ inch, 3-way valve, *C*. To drain off any water that may possibly accumulate inside the stack, a small pet-cock, *p*, is soldered flush into the base of the stack. There is likewise another pet-cock, *a*, near the base of the stack, for the withdrawal of air samples for subsequent analysis. About a foot from the bottom is a hole in the side of the stack, to which is soldered a short piece of tubing. Through this tubing a thermometer, *T*, is inserted, held in place by a rubber stopper.

Circulation of Air in Stack—To fill the stack prior to a respiration experiment with uncontaminated outdoor air and to mix the air thoroughly at the completion of the experiment a circulating device is necessary. For this purpose the elbow *A* is connected by the metal tubing *d* with the motor blower *B*, which in turn connects with the 3-way valve *C* and the nipple *b*, leading into the stack near its base. We employed a small sized Collins motor blower unit, chiefly because it was available in the Laboratory. But any rotary air impeller can be used, such as a simple, inexpensive hair drier (obviously without the heating element). In this case, however, an enlargement of the pipe between the elbow *A* and the valve *C* is necessary, to insert the hair drier and insure rapid circulation of air. A galvanized sheet iron tee of appropriate size with a removable cap would provide a suitable housing for the hair drier (air circulator) and permit accessibility, when required. A cap which seals the side outlet of the tee can be rapidly and accurately taped in place in accordance with the Lee method outlined above. The length or run of the tee is coupled onto the vertical pipe *d* and the pipe leading to the valve *C*.

Replenishment with Outdoor Air—For filling the stack with outdoor air the 3-way valve *D* is turned so as to communicate through the pipe *E* with the outdoor air, and the valve *C* is turned so as to discharge air into the room and not into the stack. To minimize resistance, the pipe *E* should preferably be connected to a 2 inch (5 cm.) galvanized sheet iron conductor pipe. Under these conditions the blower *B*, when set in motion, will discharge air directly down out of the 3-way valve *C*. This action, in turn, draws air into the bottom of the stack through the pipe *E*. Thus the entire stack can be rapidly filled with uncontaminated outdoor air. The time required to fill the stack with outdoor air will vary, depending upon the effectiveness of the blower and the speed at which it is run. With a Collins blower delivering approximately 75 liters per minute, 5 minutes are ample to secure complete renovation of the air, even when the mixed air in the stack at the end of a respiration experiment contains about 2 per cent carbon dioxide.

Temperature Relationships—The temperature of the air at the top of the stack should always be a little higher than that of the air at the bottom. Local, pronounced temperature gradients at different levels of the stack should, however, in general be avoided.

This is best accomplished by covering the entire stack, except for the top section, with some insulating material, either a blanket or the inexpensive asbestos covering commonly used around kitchen boilers. The prerequisite of having the stack warmer at the top than at the bottom is taken care of almost automatically in any laboratory, where the temperature of the air is ordinarily somewhat higher at the top of the room than it is at the bottom. To insure that the expired air enters the stack at a temperature a degree or two below that of the stack air, the expired air before entering valve *D* is passed through a bottle immersed in cold water. This bottle should not be unduly large, so as to require too long a time to sweep out the air in it.

Humidity—To aid in the stratification of the expired air and insure against its diffusion with the outdoor air in the stack, the stack air is reasonably saturated prior to the respiration experiment by the water that is always kept at the bottom of the stack. Water is introduced, as desired, from an ordinary wash bottle by removing the rubber stopper holding the thermometer. The nipple with lock nut used to hold the valve *D* at the base of the stack projects far enough into the stack (about 6 mm.) to allow approximately 150 cc. of water to accumulate at the base without flowing out of the valve. Any excess can be rapidly withdrawn by opening the pet-cock *p* which is flush with the base. Indeed, at the end of an experimental season all the water should be withdrawn by opening this pet-cock. Prior to a respiration experiment the valves *C* and *D* are turned to shut off connection with the room, and the air is continuously circulated through the stack by the blower *B*, being withdrawn from the stack through the elbow *A*, through the pipe *d*, and entering the stack through valve *C* and the nipple *b*. The discharge end of the nipple *b* is provided with a small baffle plate, which deflects the air over the water in the bottom of the stack before it rises up into the stack around the edges of the baffle plate *e*. Nearly complete saturation of the stack air is obtained by circulation of the air for about 3 minutes in this manner, either prior to the beginning of a respiration experiment or during the preliminary breathing of the subject out through the valve *D*. Complete saturation is but slowly reached, and although theoretically desirable is not necessary, as has been shown by numerous control tests, both chemical and physiological.

Stratification Versus Diffusion—By drawing a number of air

samples from the open end of valve *C* at the end of 6, 8, and 10 minutes of a respiration experiment and analyzing them for their carbon dioxide content, we found that the percentage of carbon dioxide in the issuing stack air was not significantly higher than that of the outdoor air which was in the stack at the start of the experiment. The carbon dioxide in the air at the bottom of the stack prior to the stirring or mixing of the air at the end of an experiment was usually about 3.4 per cent in the case of the subjects used in our Laboratory. After the air had been mixed, it was not far from 2 to 2.5 per cent. Thus when the air at the bottom contained 3.4 per cent carbon dioxide, the air leaving the top of the stack at the end of the 10th minute contained from 0.05 to 0.06 per cent carbon dioxide. At the 8th minute the outcoming air generally contained 0.035 per cent carbon dioxide. On the assumption that 15 liters of air were discharged from the stack between the 8th and the 10th minutes, the increase in the carbon dioxide content of the outcoming air from 0.03 to 0.06 per cent means that 4.5 cc. of the carbon dioxide from the expired air were possibly lost during the 10 minute experiment. Hence the loss of carbon dioxide from expired air is insignificant, as the total carbon dioxide production in a 10 minute period would be about 1800 cc.

Conduct of a Respiration Experiment—For the conduct of a respiration experiment the subject breathes through a mouthpiece or face mask connecting with the usual inspiratory and expiratory valves. The exhaled air is discharged from the expiratory valve, passes through the bottle immersed in cold water where it is cooled, and then passes through the pipe *F* to the 3-way valve *D* which is turned to discharge through the pipe *E* to the room during the preliminary period. At the beginning of the experiment proper the valve *D* is turned at the end of a normal expiration so that the expired air now passes directly into the base of the stack from the pipe *F*. A baffle plate, *e*, is supported 2 cm. above the floor of the stack by three small feet, which raise it a few mm. above the top of the nipple and lock nut holding the 3-way valve *D*. The exhaled air, as it enters the bottom of the stack, impinges against this baffle plate and passes around its edge and up into the stack. Meanwhile the air at the top of the stack is being slowly forced out through the elbow *A*, down the pipe *d*, and through the blower

B, which is not running at this time. As the blower is a rotary air impeller, it presents no resistance to the passage of air which finally escapes through the valve *C*, left open so that the air passes out into the room. At the end of the experiment (usually 10 minutes) and at the end of a normal expiration the valve *D* is turned, shutting off the admission of expired air to the stack, and the elapsed time precisely noted. Then the valve *C* is turned to connect with the nipple *b* in the bottom of the stack and to shut off connection with the room. The blower *B* is put in action, and at the end of 5 minutes the air in the stack is thoroughly stirred and mixed. One can then read the temperature of the air in the stack on the thermometer *T* and draw a sample of air from the stack through the pet-cock *a* for subsequent gas analysis.

Tests to Determine the Volume of Stack Apparatus and to Prove the Absence of Diffusion—The volume of the stack itself can be readily calculated from the measurements of its circumference and length, but the determination of the volume of the external circulating pipes, blower, and valves necessitates a more complicated calculation. This, however, can also be computed from direct measurements of the various parts, except for the blower *B*, which may be estimated without any significant error. If the hair drier is used instead of the blower, a somewhat larger volume is thereby introduced. Furthermore, the baffle plates and the wire netting inside the stack represent a slight reduction in volume, although almost insignificant. We used the chemical method of determining the volume of the apparatus, making it serve not only for this purpose but for a study of the stratification. A known amount of pure carbon dioxide was introduced into the bottom of the stack, which had previously been filled with outdoor air. Then the air in the system was circulated thoroughly, and an air sample was drawn and analyzed for its carbon dioxide content. From this analysis and from the known amount of carbon dioxide introduced, the volume of the system was readily calculated. Finally a mixture of air containing 3.5 per cent carbon dioxide was prepared in a small spirometer and introduced into the stack from this spirometer by means of a mechanico-chemical device simulating respiration (2). Thus quantitative carbon dioxide tests were made, from which the volume of the complete stack apparatus was found to be precisely 120.7 liters. The experience with these

carbon dioxide tests confirms the belief that the stratification is extraordinarily good and that there is a minimum amount of diffusion under the conditions under which a physiological respiration experiment is carried out.

The regular respiratory rhythm of a normal subject might be considered as presenting ideal conditions for measurements of the respiratory exchange with this type of apparatus. But pathological individuals and occasionally normal individuals oftentimes have a very irregular, forced respiration. This type of respiration is such as to throw doubt upon the real value of basal metabolism measurements made upon these individuals with any of the existing forms of respiration apparatus. With the stack apparatus a number of tests were made in which normal individuals arbitrarily engaged in intermittent and at times explosive expirations, with now and then deep inspirations. Under these conditions a slightly higher percentage of carbon dioxide was found in the air leaving the top of the stack at the end of 10 minutes, but in all instances the maximum loss of respiratory carbon dioxide, even under these extreme conditions, never exceeded 1 per cent of the total carbon dioxide exhalation.

Gas Analysis—In our tests of the accuracy of functioning of this apparatus, especially with regard to the rate of diffusion, we had the advantage of using the extremely exact Carpenter gas analysis apparatus (3). However, inasmuch as the Haldane apparatus is more generally used, it is advisable to have the final content of carbon dioxide inside the stack, after the air is thoroughly mixed, not far from 2 per cent, in order to minimize the slight inherent error of analysis with the Haldane apparatus. Consequently the experiments should be, in general, so planned as to introduce into the stack through the respiratory products not far from 2400 cc. of carbon dioxide during the experimental period. This would represent about 2 per cent of the total volume of the apparatus. If the metabolism is abnormally high, as happens in some pathological cases, one has but to shorten the length of the experimental period, which may well be decreased to 6 minutes in common with the practise of many clinics. Although we did not do so, it is feasible to have the inspired air taken from the discharge at the valve *C*, and thus any possible loss of respiratory carbon dioxide due to its diffusion into the top of the stack may be prevented.

Under these conditions a small bathing cap expansion chamber should be introduced between the valve *C* and the inspiratory valve. Even under most disadvantageous conditions, however, the diffusion is so small that it hardly seems necessary to introduce this added equipment.

Control Tests—After the volume of the apparatus was established by the chemical method of introducing into the stack a known amount of carbon dioxide, the next step was to burn a known amount of standardized alcohol in an alcohol lamp, collect in the stack the carbon dioxide resulting from the combustion, and measure the amount thus collected. Several such experiments were made, and although it is not simple to secure a gaseous mixture resulting from the burning of an alcohol flame with a carbon dioxide content as high as that in the air exhaled from the lungs, namely 3 to 4 per cent, nevertheless we were able to measure the carbon dioxide produced in these alcohol control tests to within a fraction of a per cent of the theoretical amount produced. Hence these tests proved the accuracy of functioning of the stack apparatus. Finally the apparatus was controlled by two physiological control tests. The subject breathed into the stack apparatus for three consecutive periods, each of 10 minutes. Then he was connected with what is considered the best form of respiration apparatus used in the Nutrition Laboratory for gaseous metabolism measurements, namely an open circuit type employing a helmet (4), a Fox sampling bag (5), and two dry gas meters, in conjunction with a Carpenter gas analysis apparatus for analyses of the outcoming air. With this apparatus the subject's metabolism was also measured in three consecutive 10 minute periods. Finally he was again connected with the stack apparatus for two more periods of measurement. This type of control test was carried out on 2 days. The results of these observations, which are recorded in Table I, show that on the 1st day during the first three periods when the stack apparatus was used the average carbon dioxide production per minute was 193 cc., the average oxygen consumption 242 cc., and the average respiratory quotient 0.79. The three periods with the helmet apparatus gave averages of 198 cc. for carbon dioxide, 250 cc. for oxygen, and 0.79 for the respiratory quotient. In the last two periods with the stack apparatus the carbon dioxide production averaged 197 cc., the

oxygen consumption 244 cc., and the respiratory quotient 0.81. This experiment and that on the 2nd day furnish as near as is

TABLE I
*Comparison of Metabolism Measurements with Stack and with Helmet
Respiration Apparatus*

Date and apparatus	Period No.	Per minute			Respiratory quotient
		Pulse rate	Carbon dioxide production	Oxygen consumption	
1936			cc.	cc.	
May 19* Stack	1	51	182	236	0.77
	2	52	197	242	0.81
	3	55	199	248	0.80
	Average.....	53	193	242	0.79
Helmet	4	53	196	249	0.79
	5	54	198	249	0.80
	6	53	199	252	0.79
Average.....		53	198	250	0.79
Stack	7	54	196	247	0.79
	8	55	197	240	0.82
Average.....		55	197	244	0.81
June 3† Helmet	1	54	193	239	0.81
	2	54	195	238	0.82
	3	53	200	243	0.82
	Average.....	54	196	240	0.82
Stack	4	56	197	240	0.82
	5	58	192	245	0.78
Average.....		57	195	243	0.80

* The subject had had a light breakfast.

† Subject postabsorptive.

possible a perfect physiological comparison of the two forms of apparatus, the differences between the measurements by the two apparatus not exceeding the errors inherent in the gas analyses.

Advantages of Apparatus—The chief advantage of the stack apparatus is that it has a known volume, which can be readily determined and which remains constant in all experiments. In addition to the gas analyses, the necessary observations are those of the stack temperature, the barometric pressure, and the length of the period of measurement. However, even if the temperature should fluctuate nearly 3°, this would introduce an error in the final result of but 1 per cent. Furthermore, although theoretically it is preferable to have the air in the stack saturated, the difference between complete saturation and 50 per cent saturation would affect the metabolism measurement by hardly more than 1 or 2 per cent. The apparatus is inexpensive. The stack itself can be constructed by any plumber at a cost (with the exception of the valves) of hardly 10 dollars. The Collins motor blower unit, although somewhat expensive, functions perfectly, but it can be easily replaced (as outlined above) by an inexpensive hair drier. Inasmuch as with this type of apparatus it is unnecessary to make two sets of readings with their inherent errors, either of the level of a spirometer or of gas meters, and it is unnecessary to employ the Douglas bag method from which the gas must be transferred to a metering device, this simple form of respiration apparatus should find a place in the laboratory.

SUMMARY

An open circuit respiration apparatus is described, the chief feature of which is an inexpensive chamber in the form of an elongated pipe or stack, of known and constant volume, for the collection of expired air. The principle of the apparatus rests upon the stratification of the expired air (by control of temperature and humidity conditions) at the bottom of the stack and its slow rate of diffusion with the outdoor or room air already in the stack. The subject breathes through a mouth piece connecting with inspiratory and expiratory valves. As the expired air accumulates in the bottom of the stack, it gradually expels the outdoor air from the top of the stack. At the end of 10 minutes valves are turned, making a closed circuit system of the stack, the air inside the stack is thoroughly mixed by a rotary air impeller, and a sample is withdrawn for gas analysis. From the results of such analysis, the recorded temperature of the air in the stack, the barometric pressure, the length of the period of measurement, and the known

volume of the stack, the carbon dioxide production and the oxygen consumption of the subject are readily calculated. Alcohol and physiological control tests have proved that the apparatus functions accurately to within the slight error inherent in gas analysis by the Haldane method.

It is a particular pleasure to acknowledge that Mr. William Meyenberg of the Society of American Magicians, in a most interesting effect with tobacco smoke, stimulated the basic idea underlying the development of this apparatus. It is also a pleasure to express appreciation for the invaluable aid received from my associate, Mr. Robert C. Lee, in working out the technical details of this apparatus.

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THE DISTRIBUTION OF LIPIDS IN FRESH STEER SKIN*

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The skin is not only one of the largest organs of the animal body, but its varied physiological functions and its specialized metabolism make it one of the important organs of the body. The lipids of the skin, because of their involvement in the formation of the sebaceous secretion, and their rôle in the keratinization processes of the epidermal cells, are intimately associated with the physiology of the skin. Yet comparatively few studies in recent years have been directed toward skin lipids, and our knowledge of these constituents is due to the researches of a few men.

The early work of Unna and Golodetz (21, 22) still represents the most comprehensive investigations of skin lipids. Their work was particularly concerned with the cholesterol and phospholipid distribution in human skin and with the changes these lipids undergo during skin metabolism. More recently Eckstein and Wile (7) have studied the lipid distribution of exfoliated human skin. They reported that cholesterol constitutes 13 to 24 per cent of the total lipid derived from such material, 90 per cent of which exists as free cholesterol, while phospholipid comprised only 2.5 to 3.15 per cent of the lipid obtained. The low phospholipid content of their material was undoubtedly due to the keratinous nature of the material analyzed. Roffo (19) has observed the increase in the skin cholesterol of rats subjected to ultraviolet radiation and Kawaguchi (8) has performed similar experiments

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with guinea pigs and rabbits. Pachur (18) has analyzed the lipids of the human skin surface. The recent papers of Kooyman (9, 10) have reported the changes which the epidermal lipids undergo during keratinization and the character of the surface lipids of the human skin.

It has been difficult in attempting to study the lipids of the human skin to obtain normal tissue in sufficient quantity for analysis. The investigators have, at times, resorted to peculiar sources to obtain lipids representative of various skin structures or of various skin processes. The use of the skin of the steer in studying the metabolism of skin lipids offers obvious advantages. It can be obtained in a normal state immediately after slaughter and postmortem activity can be reduced to the time required to prepare the material for alcoholic dehydration. Furthermore, it is of such a quantity and thickness that it can be separated mechanically into representative horizontal divisions. A study of the distribution and character of the lipids of these divisions yields, as will be seen, much information concerning the general lipid metabolism of the skin.

A previous publication (11) has indicated the nature and distribution of the various lipids of the steer skin. The epidermal region was found to contain the preponderance of the phospholipids and cholesterol. In addition, a wax composed of long chain, saturated hydroxy acids in ester combination with aliphatic alcohols was isolated in this region. The corium contained smaller amounts of phospholipid and cholesterol but varying and often large deposits of triglycerides similar in chemical nature to those contained in the reserve lipid deposits of the subcutaneous tissue. It was evident from the data obtained that a striking similarity existed between the lipids of human skin and those of the steer skin, particularly in regard to their nature and distribution.

Based upon some preliminary tests correlated with histological evidence, the separation of the following divisions seemed desirable to illustrate the metabolic activity of the skin lipids. After the hair was clipped, the skin was divided into six horizontal divisions, each representing loosely defined skin structures. The separation is shown diagrammatically in Fig. 1. The analysis of lipids of the subcutaneous layer, having been reported previously, was not attempted.

The uppermost division, consisting of the hair, needs no description. The next division, termed the "horn" because it embraces that portion of the epidermal system resulting from the keratinization processes, together with the adhering surface lipids, contains the end-products of the lipid metabolism of the epidermal region. The adjacent division, the "basal" division, includes the region extending from the nucleated epidermal cells to the base of the hair roots. The mechanical separation of this division is easily possible because the hair roots afford a sharp contrast to the white back-

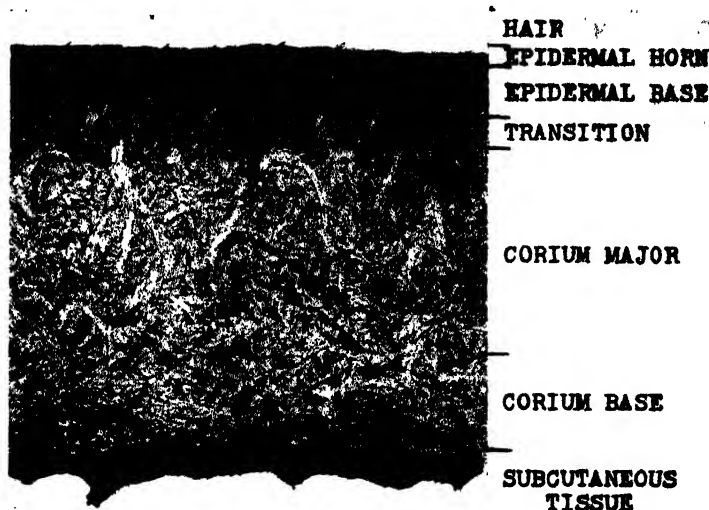


FIG. 1. Section of normal steer skin, illustrating the separation of the various divisions. Stained with scarlet R; $\times 7.5$.

ground of the underlying material. This division contains the following structures—the hair pockets, the sebaceous and sudoriferous glands, the nucleated cells of the stratum germinativum, together with the preponderance of the wandering cells interspersed throughout the corium minor. The histological structure of this division, as well as the chemical analyses of its lipid, indicates it to be a region of comparatively intense physiological activity. The next division removed constitutes the upper portion of the corium adjoining the base of the deepest hair roots. It will be referred to as the "transition" division since its removal

insured a complete separation of epidermal structures from the corium division. It is in this region that the transition of the connective tissue structure from the fine fibers of the corium minor to the large fiber bundles of the corium major occurs. Furthermore, preliminary experiments indicated that this region contained a sphingomyelin-like lipid which is present in some skins.

The corium was separated into two divisions, the corium major and the corium base. The corium major comprised 60 per cent of the dry weight of the skin. It is composed, mainly, of bundles of white connective tissue fibers, together with smaller quantities of reticular tissue sheaths and interfibrous material. The solidity of this region and the intricate manner in which its constituent fibers are interwoven to give the skin the physical toughness it possesses make this the most important leather-producing region of the skin. The lower corium division, or corium base, differs from the upper corium division particularly in the increased concentration of fat cells which are present.

EXPERIMENTAL

The skin of a 2 year-old, grade steer was obtained immediately after slaughter and cut into strips parallel to the median line. Each strip was mechanically separated with a sharp razor into the various divisions outlined above. Obviously the precision of such a separation is limited by the variable structure of the skin. However, with careful technique and close observation of the structural demarcations of the various divisions it is possible to obtain satisfactorily consistent separations. This was further checked by microscopic examination of the various divisions. This method is preferable to any chemical means of separation since it avoids changes in the character of the lipids due to their reactivity.

The material obtained was cubed, if necessary, and plunged into cold ethyl alcohol. The average time elapsing between slaughter and alcohol immersion was 3 hours. After complete dehydration had been obtained through several changes of alcohol, the material was ground in a Wiley mill and extracted with successive changes of hot alcohol until negligible amounts of lipids remained. The completeness of the extraction was further tested by hydrolyzing

a portion with strong alkali, acidifying, and extracting the acidified solution with ethyl ether. A previous publication from this laboratory (15) has attributed the failure completely to remove, by solvent extraction, all the lipid from the steer skin to the presence of a lipid-protein complex. With more adequate facilities for subdivision and extraction of hide material, it has been possible, with solvent extraction, to remove 98 per cent of the total skin lipid. On filtering the hot alcoholic epidermal extracts, a slightly soluble wax separated from the warm alcohol. In order to obtain a maximum separation of this wax, the epidermal extracts were cooled to 5° overnight and the wax recovered by filtration. The respective alcoholic solutions of the lipids, including the dehydration solutions, were evaporated in a vacuum still and the residual lipids almost completely recovered by solution in ethyl ether. Weights and iodine numbers were then determined on aliquots of the ether solution. The ether-soluble lipids were further fractionated, after removal of the ethyl ether, by twice dissolving them in about 750 cc. of hot alcohol and allowing the less soluble lipids to precipitate overnight at 5°. By this procedure, most of the complex lipids, as the cholesterol and phospholipids, remained in solution in the cold alcohol, while the triglycerides of the corium and the remaining waxes of the epidermis were obtained as insoluble material. The phospholipids were then removed from the cold alcohol-soluble lipids according to Bloor's procedure (3). In this manner five fractions were obtained, diluted to volume, and aliquots removed for analysis. They were the waxes, the cold alcohol-insoluble lipids, the alcohol-acetone-soluble lipids, lecithin, and cephalin.

The usual constants were determined on these fractions by the standard procedures of the American Association of Agricultural Chemists (16). Free cholesterol was determined colorimetrically by a modification of the Liebermann-Burchard reaction (12), while cholesterol ester was determined by the method of Bloor and Knudson (5). Hydroxycholesterol was approximated by application of the Lifschütz reaction.

Results

From a summation of the detailed analyses of various fractions presented in Tables II to VII, a compilation of the more significant

variables was prepared. These are presented in Table I. These data, together with the analyses of the various fractions, demonstrate not only the character and distribution of the various lipid entities, but also indicate the processes of the lipid metabolism in the skin.

Distribution of Lipids in Skin—The data presented in Table I show that the lipid concentration is greatest toward the upper and lower extremities of the skin. In the epidermal region this deposition of lipid consists of the phospholipids, cholesterol, and waxes, while the increased lipid content of the corium base is due to the triglyceride deposition. The subcutaneous fat tissues were carefully removed before the mechanical separation and they are not to be confused with the deposition of lipid within the corium. Histological examination of skin sections demonstrates, in a qualitative manner, that such a distribution of lipid exists in the skin.

Triglycerides of Corium—The preponderance of the lipids of the corium, demonstrable by histochemical technique, occur as cellular deposits distributed among and between the corium fiber elements. This deposition has been shown (17) to be more pronounced toward the subcutaneous layer and to be identical in histological structure to the cells of this layer. The analyses of the corium lipids, recorded in Table I, show that the corium base contains 7.15 per cent lipid and the corium major 2.44 per cent lipid, 77 to 80 per cent of which is cold alcohol-insoluble triglycerides. The quantity of such triglycerides isolated from the corium is approximately proportional to the histologically demonstrable lipid in the corium. Both chemical data and histological evidence indicate the increased deposition of this material toward the subcutaneous region, and the analyses of these triglycerides and the lipid of the subcutaneous deposits are almost identical. Hence these lipids result from the deposition of triglycerides in the skin as well as in the subcutaneous lipid reserves. The amount of such deposits varies considerably from skin to skin, depending evidently upon the nutrition of the animal. When present in large amounts, such lipids are the major factor in the production of detrimental grease stains often found on finished leather.

The chemical characteristics of the corium triglycerides are listed in Table II. The separation of the fatty acids by the lead salt-ether procedure shows that they consist of 65 per cent liquid

acids and 35 per cent solid acids. The liquid acid is oleic acid and the solid acids are mixtures consisting principally of palmitic and stearic acids. Other workers have determined the constituent acids of tallow prepared from this material (2). We have isolated a saturated triglyceride which appears to be tripalmitin from these corium lipids (11).

Cold Alcohol-Insoluble Lipids of the Epidermis—The precipitation in cold alcohol of the lipids derived from the epidermal divisions results in the separation of a mixture of several different

TABLE II
*Analyses of Cold Alcohol-Insoluble Fractions**

	Epidermal region		Corium region	
	Horn	Basal	Corium major	Corium base
I No	15.0	43.6	54.7	51.8
Acid value	5.0	6.3	0.2	0.2
% fatty acid	60.7	61.0	93.2	92.4
Mean mol. wt.	323.0	301.5	272.8	274.1
I No	10.2	45.9	55.6	53.6
Saponification No	187.6	199.0	209.6	209.4
Acetyl value	82.6	37.8	0.0	0.0
% unsaponifiable	40.6	35.1	1.7	2.0
I No	25.1	40.9	55.1	59.0
Acetyl value	191.5	178.2		
% cholesterol	14.9	41.1		
% lipid phosphorus	0.0	0.56	0.0	0.0

* This fraction was not isolated from the lipids of the hair and transition divisions because of the small quantity of total lipid available.

types of lipids. The epidermal lipids of this fraction consist of the more soluble waxes, cholesterol esters, and small amounts of phospholipids and triglycerides. The waxy nature of the fraction from the horn division is particularly evident. The alcohols of these waxes, judging by the acetyl value of the non-saponifiable material, must be C₁₄ and C₁₆ alcohols, which probably accounts for their failure to precipitate with the less soluble wax fraction. The cholesterol of this fraction is 80 to 100 per cent esterified. The fatty acids from the horn division, present in this fraction, are similar to those of the wax fraction, while those of the basal division show accentuated oxidation characteristics.

Phospholipids of Skin—The lipid phosphorus distribution in the skin is likewise presented in Table I. The phospholipid distribution in the two corium divisions is constant. This is particularly significant in view of the disproportionate distribution of triglyceride among these divisions. The corium phospholipid content of many skins examined in other phases of this work has been observed not to follow the triglyceride content of the corium but to remain fairly constant. It is evidently associated with the fundamental protoplasmic structure, the fibroblasts of the corium. Phospholipids appear to be a necessary constituent of living cells and Bloor (4) has correlated the degree of physiological activity of the cells with their phospholipid content. The passive activity of the corium region as compared to the epidermal region might well be associated with the difference in their phospholipid content.

The rapidly multiplying cells of the stratum germinativum of the epidermal layer and the wandering cells which are found in the region directly beneath the epidermal layer exhibit characteristic staining reactions with basic dyes which are attributed to the acidic constituents of the cells. These cells, particularly those of the epidermal layers, contain large numbers of mitochondria. The studies of Mayer, Rathery, and Shaeffer (14) have shown the similarity between the chemical and physical properties of the cell mitochondria and the phospholipids. The acidic nature of the phospholipids may explain, in part, the characteristic stain reaction of these cells with basic dyes. The mitochondria are active in the metabolic processes of the cell and their number can be correlated with the degree of physiological activity of the cell, as can the phospholipid content.

The lipid phosphorus concentration in the epidermal region as recorded in Table I reaches a maximum in the basal epidermal region. This is also the region in which the nucleated cells of the epidermal layers show their maximum development. There is an appreciable decrease in lipid phosphorus in the horn division and none was identified in the lipid isolated from the hair. It is evident that the phospholipid molecule is destroyed during keratinization. The analyses of the phospholipid fractions in Table III record the gradual decomposition of the phospholipids of the epidermal cells.

The cells of the sebaceous glands are originally derived from the

epidermis and they likewise undergo a degenerative process with the resultant formation of the sebum. The analyses of the basal epidermal region indicate that phospholipid and free cholesterol must be the principal lipid constituents of these active cells. Using the Golodetz reaction on human skin, Unna and Golodetz (21) found that most cholesterol was contained in the embryonic cells of the epidermis, the hair pockets, and the sebaceous glands. The large quantity of lipid which the cells of the sebaceous glands accumulate during degeneration is deposited within the sebaceous gland to form the essential constituents of the sebum. The analysis of this sebum, which will be discussed later, shows that it is

TABLE III
Analyses of Phospholipid Fractions

	Lecithin			Cephalin
	Epidermal region		Corium major	Basal
	Horn	Basal		
Lipid, gm	0.89	6.54	1.29	1.27
I No	60.6	67.7	71.5	72.4
% fatty acid	59.7	68.4	61.6	79.4
Mean mol. wt.	398.5	360.0	347.0	412.5
I No	66.5	77.2	83.0	90.4
Saponification No.	191.5	171.7	183.5	196.0
Acetyl value	74.9	52.1	51.2	45.9
% unsaponifiable	13.0	8.5	7.0	7.7
I No	45.4	77.1	102.3	77.2
% cholesterol	4.4	6.4	1.0	1.8
% lipid phosphorus	2.83	3.82	3.85	2.90

composed of cholesterol esters and waxes of hydroxylated fatty acids in ester combination with aliphatic alcohols. In this respect, the hydroxylated nature of the phospholipid fatty acids is significant in indicating that they are the probable source of the acid constituents of the wax described above. Evidently the transformation of the cholesterol and phospholipid of epidermal cells, occurring during the degenerative processes resulting in sebum formation, is effected by a concurrent hydroxylation and esterification of the lipid constituents to form the characteristic waxes of the sebaceous secretion. The manner of formation of the aliphatic alcohols present in the sebum waxes is uncertain.

The phospholipids represent an easily separated entity in the various divisions and the analyses of the lecithin fractions of the epidermal divisions show the changes which are effected in these lipids during keratinization. Particularly significant are the decreases in the iodine number and the increase in the hydroxylation of the constituent fatty acids recorded in Table III. Sufficient lecithin was not obtainable to permit complete analyses in all the divisions. Cephalin was obtained in quantity sufficient for analysis only from the basal epidermal division. The cephalin is more unsaturated than the lecithin and its fatty acids are of higher molecular weight. The high molecular weight of the acids of the phospholipids, calculated from their alkali equivalence, indicates that polymerization or lactone formation occurs. Saponification following the titration of the fatty acids in hot alcohol resulted in an increased alkali absorption as these complexes are broken down. Only liquid fatty acids are separated by application of the lead salt-ether procedure to the phospholipid fatty acids. The double precipitation procedures employed in the separation of the phospholipids failed to remove an appreciable amount of the non-saponifiable material. The preparation of pure lipid entities was not considered necessary in the evaluation of the general characteristics required.

Cholesterol of Skin—The work of the early German investigators, cited in the review of Rothman and Schaaf (20), as well as the recent publications of Eckstein and Wile (7) and Kooyman (9, 10) have associated the greater proportion of the skin cholesterol with the epidermal region. Particular attention has been devoted to the cholesterol esterification which is believed to occur during keratinization of the epidermal cells. The presence of cholesterol in the sebum is disputed.

The total cholesterol distribution is given in Table I. A more detailed examination of the various forms of cholesterol and their distribution was made upon a second skin. These results are presented in Table IV. The corium cholesterol, like the lipid phosphorus, is uniformly distributed throughout the corium. The close association between cholesterol and the phospholipids in living tissues has led to hypotheses concerning their mutual physiological relation. The uniform distribution of cholesterol in the corium and the constancy of its weight relation to the dry

corium weight, as observed in several skins, suggest that it, like the phospholipid molecule, is associated in the cellular elements of the corium. The cholesterol of the corium exists in the uncombined state.

The increase in the cholesterol content beginning in the transition division and reaching a maximum in the epidermal region follows in part the phospholipid distribution in the same regions. Thus the cholesterol and phospholipid distribution, as illustrated by the phosphorus-cholesterol ratio, shows some degree of uniformity in those divisions in which keratinization processes are absent. However, the decrease in phospholipid in the epidermal horn division is not accompanied by a concurrent decrease in cholesterol, and cholesterol but no lipid phosphorus was present

TABLE IV
Cholesterol Distribution (Determined on a Second Skin)

	Epidermal region	
	Horn	Basal
Cholesterol, gm.	2.46	2.68
% cholesterol in dry material	1.62	0.92
Esterified cholesterol, gm.	0.81	1.04
% cholesterol as ester in dry material	0.54	0.36
% " esterified	33.1	38.8
Hydroxycholesterol test.	Positive	Negative

in the lipids of the hair division. This indicates that a more rapid destruction of phospholipid than of cholesterol occurs at the skin surface. Kooyman (10) has also observed this decrease in phospholipid during keratinization in human skin, but he observed a concurrent, less rapid removal of cholesterol, a characteristic which these results do not show. The high cholesterol content of the skin surface, resulting because of its more stable nature, must evidently depend upon those factors which would tend to retain an intact surface, such as long hair. This would explain the variations in the cholesterol content of the horn layer of the two skins as expressed in Tables I and IV.

A considerable percentage of the epidermal cholesterol exists as cholesterol ester. The identification of only combined cho-

lesterol in the sebaceous secretion (see Table V) accounts for its presence in the epidermal divisions. Since the sebum is the predominant source of surface lipids, it is probable that the preponderance of the cholesterol esters on the surface arises from this source. Whether cholesterol esters are likewise produced during keratinization cannot be concluded from these experiments. The identification of hydroxycholesterol in the horn division and its absence in the underlying basal division suggest the manner of cholesterol destruction on the surface.

The free cholesterol was isolated principally from the acetone-alcohol-soluble fraction, where it may comprise as high as 80 per cent of the non-saponifiable material. The cholesterol esters, because of their lesser solubility, are present in the cold alcohol-insoluble fraction and in the wax fraction where they represent the total cholesterol present. It is difficult to remove the cholesterol completely from the phospholipid fractions.

Waxes of Epidermis—A previous publication has reported the presence and character of the waxes present in the epidermal region of the steer skin. Linser (13) has reported the presence of such waxes on the human skin surface, and Ameseder (1) has isolated *n*-eicosanol (arachyl alcohol) from such a source. We have isolated the same alcohol from the epidermal waxes of the steer skin. Because of the saturated nature of the wax and its distribution, it was first believed to result from oxidation processes at the epidermal surface, and to constitute the waxy sheath which protects the skin surface. However, in this experiment, the horn division contained only 30 per cent of the total wax present, while the basal epidermal division contained 65 per cent of the total wax. This definitely demonstrated that this wax was not entirely of surface origin.

The procedure with a second skin was slightly altered to determine the reason for such a distribution. After removal of the horn division the sebaceous glands were clearly visible, imbedded just below the surface. By rotating the razor so that scraping instead of cutting action was attained, it was possible to press the oily glandular secretion to the surface, where it could be collected. Histological sections of the scraped skin showed that a definite removal of sebaceous lipid was obtained by this procedure. It is significant that lipid was pressed from the glands only when the

force was applied in the direction of the grain; *i.e.*, with the slope of the hair follicle.

The analyses of this lipid, presented in Table V, as compared to the analyses of the total lipid of the entire basal division from which it was expressed, indicate the nature of the unaltered sebaceous secretion. Obviously, material separated in such a manner would be contaminated to a small extent by the lipids of the cut surface which might be picked up during the removal of this fraction. Fortunately certain pronounced differences exist between the lipid removed from the gland and that of the basal division, from which the extent of such contamination might be judged. Thus the esterified nature of the cholesterol of the

TABLE V
Analysis of Sebaceous Secretion (As Isolated)

I No	32.6
• Acid value	10.1
Saponification No.	154.8
% fatty acid	57.4
Mean mol. wt.	249.1
I No.	27.3
Acetyl value	74.9
% unsaponifiable	42.7
I No	36.0
% total cholesterol	14.4
% esterified cholesterol	13.7
% lipid phosphorus	0.159

sebaceous secretion compared to the high free cholesterol content of this division, the comparatively low free fatty acid content, and the low lipid phosphorus content of this lipid as compared to the lipid of the basal division are significant factors in indicating the small degree of contamination of the sebaceous lipid isolated. The analysis of the secretion, recorded in Table V, shows the nature of its lipid contents. A direct comparison with the saturated wax of the basal epidermal division (see Table VI), from which the cholesterol ester has been removed, indicates that the sebum contains two distinct types of waxes. The one group comprises the aliphatic alcohol, saturated hydroxy acid esters which are present in great quantities in the epidermal region. The other

group is the esters of cholesterol and evidently more unsaturated acids. This latter group would be effective in producing the iodine numbers recorded for the various lipid fractions. One recrystallization from alcohol removed the free fatty acids and lipid phosphorus, which contaminated the lipid, along with some of the constituent cholesterol esters and left a waxy residue constituting 75 per cent of the lipid pressed from the skin. It seems certain then that the origin of the epidermal wax is the sebaceous secretion and that this secretion is composed of the esters of fatty acids and aliphatic alcohols or cholesterol to yield a low melting, waxy material.

TABLE VI
Analyses of Wax Fractions

	Epidermal region		
	Hair	Horn	Basal
I No	9.3	4.9	6.5
Saponification No.	149.5	156.0	175.6
Acid value.	1.3	0.9	0.3
% fatty acid.	75.4	59.2	55.5
Mean mol. wt.	361.0	261.0	252.0
I No		3.9	5.3
Acetyl value.	91.1	85.4	79.2
% unsaponifiable.	26.9	44.1	45.4
I No		6.2	7.4
Acetyl value.		182.2	175.0
% cholesterol	7.2	6.2	8.9

The analyses of the waxes isolated from the three epidermal divisions are presented in Table VI. The cholesterol of this fraction is completely esterified and its partial separation in this fraction is due to the insolubility of such compounds. From such waxes we have already reported the isolation of isohydroxystearic acid, stearic acid, and *n*-eicosanol (arachyl alcohol) (11). The analyses of the non-saponifiable material suggest the presence of lower chain alcohols other than the C₂₀ alcohol isolated. The fatty acids have been subjected to fractional distillation and the results, yet incomplete, indicate that shorter chain acids of the saturated series, probably lauric and myristic, are also present.

It is not possible to conclude from these experiments whether the disintegration of the epidermal cells represents an additional source of waxes. A slight difference exists between the character of the waxes of the basal and horn divisions, which might be accounted for by such an additional location of wax formation in the epidermis. The concentration of waxes on the surface of several skins has been found to vary in the same relation as the surface cholesterol, with which they are associated. The wax concentration of the basal division depends upon the sebaceous activity of the animal.

Free Fatty Acids of Skin—The high free fatty acid content of the lipids of the human skin surface has been reported by Kooyman (10), who attributed it to the lipolytic action of bacteria and enzymes and to atmospheric oxidation at the skin surface. The data in Table I show the progressive increase in free fatty acid toward the surface of the skin. In an attempt to associate the increase in free acid with the concurrent decrease in phospholipid, the free fatty acids were isolated. The analyses of the acids of the horn division were similar to those of the alcohol-acetone-soluble fraction except that the free acids had a slightly higher acetyl value. No conclusion could be made as to the origin of the acids, but that some hydroxylation occurred after hydrolysis seemed evident. The free fatty acids of the basal division, however, vary considerably in character from the combined acids of this region. They possess an acetyl value of 56.2 compared to 25.6 for that of the combined acid of the acetone-soluble fraction and 34.8 for the phospholipid fatty acids. The mean molecular weight of 311 of the free fatty acids compares with a molecular weight of 270 for the combined acid. These characteristics tend toward those of the acids of the sebum waxes and it is possible that these acids of the basal division are intermediates of the wax metabolism occurring in the sebaceous glands.

Composite Iodine and Acetyl Values—After separation of the insoluble waxes, all the alcohol used for extraction was evaporated and the lipid taken up in ethyl ether. The iodine numbers of these ether-soluble lipids were determined and they are recorded in Table I. The iodine numbers of the corium lipids are nearly identical with those of the corium triglycerides which represent the preponderance of the corium lipids. The increase in iodine

number in the transition and basal divisions reflects, as does the phospholipid distribution, the state of increased activity existing around the sebaceous glands. The decreased iodine number of the lipids of the horn division is the result of oxidation occurring during keratinization.

The hydroxylation of the fatty acids of the various divisions is presented. Since the fatty acids of the corium triglycerides are not hydroxylated, the acetyl value of the acids of the corium lipids is due to the acids of the phospholipid and alcohol-acetone-soluble fractions. There is a decided increase in the acetyl values of the

TABLE VII
Analyses of Alcohol-Acetone-Soluble Fractions

	Epidermal region			Transition	Corium region	
	Hair	Horn	Basal		Corium major	Corium base
I No.....	48.4	51.8	65.0	59.6	65.3	65.9
Acid value.....	66.8	75.6	28.5	24.7	22.0	21.8
% fatty acid.....	56.2	57.5	45.6	61.2	61.5	78.5
Mean mol. wt....	271.5	270.3	286.0	309.0	280.2	277.0
I No.....	41.4	49.5	65.1	60.6	69.1	67.4
Saponification No..	215.1	218.8	213.5	186.0	207.4	207.8
Acetyl value.....	22.6	21.7	42.2	37.8	9.7	8.4
% unsaponifiable...	38.1	33.7	43.6	24.5	25.1	13.4
I No.....	56.1	63.5	68.1	65.0	73.8	80.1
Acetyl value.....	159.3	167.0	145.5	157.8	156.9	145.1
% cholesterol.....	44.0	49.6	79.1	47.5	49.0	41.4
% total cholesterol..	16.8	16.7	34.5	11.6	12.3	5.55
% lipid phosphorus.	0.05	0.30	0.82	0.77	0.22	0.21

transition and basal division acids. The composite value varies with the activity of the sebaceous glands and with the amount of sebum formed. However, the acetyl value of the wax-free, ether-soluble lipids is also a maximum in the basal division. Such hydroxylation is certainly associated with the sebum formation in yielding the waxes which comprise this secretion. The phospholipids represent a definite skin entity whose evolution to the surface can be traced. A comparison of the values in Table III indicates that concurrent hydroxylation and an increased saturation occur in the phospholipid fatty acids during keratinization. It is

impossible to make such a comparison with the lipids of other fractions or from the composite values recorded in Table I because of the varied distribution and solubility of the sebum waxes.

Analysis of Alcohol-Acetone-Soluble Lipids—Those lipids remaining after the completion of the fractionation procedure constitute the alcohol-acetone-soluble fractions, the analyses of which are presented in Table VII. In this fraction are found the free fatty acids, the free cholesterol, some cholesterol esters, an appreciable percentage of acetone-soluble phosphorus-containing lipids, and the alcohol-soluble triglycerides. An application of Channon and Foster's procedure (6) for the isolation of phosphatidic acid salts from 3 gm. of this lipid resulted in the isolation of 33 mg. of lead phosphatide, indicating that the acetone-soluble phosphorus cannot be due to the presence of phosphatidic acid. From the hair and transition divisions, the lipids of this fraction constitute the total ether-soluble lipids of the division.

Analysis of Transition Division Lipids—The transition division was isolated to insure a complete separation of epidermal and corium material. Furthermore, it was thought that this division, comprising 7 per cent of the dry weight of the skin, might contain a sphingomyelin-like lipid fraction which has been isolated from some skins and which appears to exist in this region. Its absence in this skin, an irregularity which has been previously observed with other skins, leaves this point undetermined. The analysis of the lipid of this fraction is interesting, for it indicates by its character a transition from corium to epidermal lipid. Particularly significant is the increase in lipid phosphorus and cholesterol as lipid constituents and the higher iodine number and acetyl value as compared to the lipid of the corium material. Whether this is characteristic of this region or the result of insufficient separation of epidermal material is not clear.

Analysis of Lipid of Hair—The lipid material adhering to the hair is similar to that of the horn division from which it is undoubtedly derived. Such differences as do exist between these two divisions consist in the total disappearance of lipid phosphorus and the continued increase in the free fatty acids as a constituent of the lipid of the hair. The presence of cholesterol suggests its stability as compared to the phospholipid molecule. The cholesterol ester content of the hair was not determined.

SUMMARY

A study has been made of the lipids of the skin of the steer. The work has been directed toward examining the general nature and distribution of the lipid constituents of the skin. This has been facilitated by a mechanical separation of six approximate horizontal layers of the skin. The following conclusions may be drawn.

The lipids of the corium may be classified into two groups, one composed of the complex lipids and sterols, the other comprising the triglycerides. The former are associated in the skin as active constituents of the protoplasm and they are intimately involved in the physiological activity of the tissue. The uniform distribution of the phospholipids and cholesterol in the corium region, in spite of large variations in the triglyceride distribution, is in accord with such an interpretation. The triglycerides of the corium are deposited in fat cells which are more concentrated in the region near the subcutaneous tissues. Their quantity varies considerably with the individual skin and is evidently influenced by the diet of the animal.

Whereas the triglyceride content of the corium is characteristic of that region, the wax deposition is equally characteristic of the epidermal region. In addition, the complex phospholipids and cholesterol constitute a high percentage of the lipids of this region. The sebaceous glands and the nucleated epidermal cells represent the two major sources of lipid in this region. The lipids from these two sources collect upon the epidermal surface where they serve as a protective agency for the skin.

The "basal" epidermal division is the site of the skin's most active metabolic processes. In this region the sebum results from the degeneration of the nucleated cells which line the sebaceous glands, and the phospholipids and cholesterol of these cells are transformed into the waxes and cholesterol esters of the sebum. The sebum is exuded upon the skin surface. During keratinization of the epidermal cells the lipids of these cells undergo a concurrent degeneration, the suggested nature of which appears similar to that occurring in the sebaceous glands. A differentiation between two such processes involving degeneration of epidermal cells is not possible from this experiment but the results do indicate the lipid degeneration in the horn division. Thus we have

shown the destruction of the phospholipids, the accumulation and gradual oxidation of cholesterol, and a slight change in the character of the waxes of this "horn" division. Increased saturation, hydroxy acid formation, and the liberation of free fatty acid at the skin surface have been demonstrated. Hydroxycholesterol is present in the epidermal horn division but not in the underlying basal division.

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THE RATE OF PROTEIN FORMATION IN THE ORGANS AND TISSUES OF THE BODY

I. AFTER CASEIN REFEEDING*

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During a fast the total amount of protein disintegrated is not the sum of equal contributions from all parts of the body. Some organs and tissues lose a great deal of their protein, some a smaller proportion, and others none at all (1). Such inequalities in protein catabolism raise the question as to whether each organ and tissue may not also maintain its own individual character with respect to the processes of protein anabolism. So in this paper we give measurements of the quantities of new protein formed in the various organs and tissues when casein is given after a period of loss induced by protein deprivation, and, as these measurements were made at successive intervals of time after the casein was administered, they indicate the rate of protein formation in different parts of the body.

The reformation of protein was observed under three different conditions: first, when casein alone was given after a 7 day fast; second, when it was fed with vitamins and fats; and third, when it was administered as part of a complete diet after 10 days on food almost free from protein but adequate in all other respects. The same general environmental conditions described in preceding papers were observed. Each estimate represents the average protein per rat of one or more groups of thirty male rats between 90 and 110 days of age. Altogether the protein of twenty-four such groups was determined, but since the results

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obtained under the same conditions were closely alike only one average is given for each period of observation. The initial body weights of all these groups were not identical and the actual measurements have been corrected for such inequalities by calculating what they should have been if all the groups at the time they commenced the period of protein depletion had had an average weight of 200 gm. (310 sq. cm. of body surface). The protein was determined by the gravimetric methods recently described (2) with certain exceptions to be mentioned later.

EXPERIMENTAL

In the first experiment the casein was given in the form of a suspension of a commercial casein preparation in water and tragacanth (casein 200 gm., tragacanth 12 gm., sodium benzoate 1.2 gm., with water to 1000 cc.). Groups fasted for 7 days were killed 2 days and 7 days after the commencement of feeding. In Table I the quantities of protein found in certain organs and tissues are contrasted with the quantities found after 7 days of fasting. These values for fasted rats which form the base line for this and for the next experiment are derived from data already given in detail elsewhere (1).

In the second experiment the casein was given with vitamins and fats (casein 700 gm., lard 150 gm., sardine oil 100 gm., alfalfa powder 20 gm., and dry yeast 30 gm.).

In the last experiment the quantities of protein found after 10 days on a diet almost free from protein were taken as the base line (corn-starch 740 gm., cod liver oil 100 gm., yeast 100 gm., alfalfa powder 20 gm., and salt mixture (Osborne and Mendel¹) 40 gm.). The observations were made on groups killed at intervals of time after they had been given a diet similar to the above, except that an air-dry commercial casein replaced the corn-starch. This experiment was done in 1932 and 1933 at a time when a gravimetric method had been worked out only for the liver and kidney. In some of the earlier observations on the liver there are no glycogen corrections and these had to be estimated from other determinations. At that time the protein of the heart and carcass was calculated from the N₂ content. An attempt was

¹ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.* **37**, 572 (1919).

made to wash out as much non-protein nitrogen as possible from the air-dry powdered carcass. But such substances as creatine

TABLE I

Quantities of Protein Found after 7 Days of Fast Contrasted with Those Found after Feeding Casein

	7 days fasted	+ 2 days casein feeding		+ 7 days casein feeding	
	Protein	Protein	Gain or loss from fasted	Protein	Gain or loss from fasted
	gm.	gm.	gm.	gm.	gm.
Liver.....	0.92	1.30	+0.377	1.30	+0.376
Kidney.....	0.174	0.211	+0.036	0.241	+0.066
Heart.....	0.098	0.113	+0.014	0.094	-0.004
Drawn blood.....	0.95	0.82	-0.123	0.94	-0.006
Carcass.....	26.7	28.65	+1.934	25.74	-0.976
Total gain.....			2.361		0.442
“ loss.....			0.123		0.986
Balance.....			+2.237		-0.544

TABLE II

Quantities of Protein Found after 7 Days of Fast Contrasted with Those Found after Feeding a 70 Per Cent Casein Diet

	7 days fasted	+ 2 days 70 per cent casein diet		+ 7 days 70 per cent casein diet	
	Protein	Protein	Gain or loss from fasted	Protein	Gain or loss from fasted
	gm.	gm.	gm.	gm.	gm.
Liver.....	0.92	1.43	+0.505	1.47	+0.554
Kidney.....	0.174	0.215	+0.040	0.246	+0.071
Heart.....	0.098	0.102	+0.004	0.106	+0.008
Drawn blood.....	0.95	0.89	-0.058	0.95	+0.001
Remainder.....	1.54	1.97	+0.427	1.98	+0.437
Carcass.....	26.7	28.3	+1.626	27.4	+0.678
Total gain.....			2.602		1.749
“ loss.....			0.058		0.0
Balance.....			+2.544		+1.749

cannot be so removed from the interior of coagulated muscle particles and, since the N_2 concentration of many such substances is high, an appreciable error is introduced. This error, it is true,

TABLE III
Quantities of Protein Found after 10 Days of 0 Per Cent Casein Diet Contrasted with Those Found after Feeding a 74 Per Cent Casein Diet

	10 days 0 per cent casein diet		+ 0.5 day 74 per cent casein diet		+ 0.7 day 74 per cent casein diet		+ 2 days 74 per cent casein diet		+ 5 days 74 per cent casein diet		+ 10 days 74 per cent casein diet	
	Protein	gm.	Protein	Gain or loss gm.	Protein	Gain or loss gm.	Protein	Gain or loss gm.	Protein	Gain or loss gm.	Protein	Gain or loss gm.
Liver.....	1.20	1.27	+0.064	1.57	+0.373	1.80	+0.598	1.84	+0.641	2.10	+0.894	
Kidney.....	0.193	0.204	+0.011	0.231	+0.038	0.225	+0.032	0.268	+0.074	0.301	+0.108	
Heart.....	0.124	0.126	+0.002	0.139	+0.015	0.133	+0.009	0.130	+0.006	0.141	+0.017	
Carass.....	28.36	28.31	-0.047					29.50	+1.094	30.58	+2.233	
Total gain.....			0.078					1.815			3.241	
" loss.....			0.047					0.0			0.0	
Balance			+0.031					+1.815			+3.241	

exists also in gravimetric determinations, but, because the molecular weight of these impurities is small relative to the molecular weight of the proteins, it becomes much less considerable. For these reasons we do not base any conclusions on the absolute values for the carcass and heart and present them only as indicating the direction of the relative changes induced by the change in dietary conditions.

DISCUSSION

The results given in Tables I to III show that in the rebuilding as in the disintegration of protein there is no uniformity in the absolute or relative anabolism of various parts of the body. Each organ and tissue has its own individual tempo with respect to the processes of reconstruction. There is even an instance, on

TABLE IV
Percentage Distribution of Total Body Protein

	Liver	Kidney	Heart	Other organs	Carcass
Fed.....	4.5	0.64	0.35	9.5	85
2 day fast.....	3.7	0.65	0.36	9.0	86
7 " ".....	3.3	0.57	0.32	8.2	88
+ 2 days 70 per cent casein...	4.3	0.65	0.31	8.7	86
+ 7 " 70 " " " " ...	4.6	0.76	0.33	9.1	85

the 7th day of casein feeding, in which in spite of a net loss of protein from the body as a whole the liver maintains its gain and the kidney continues an increase in its protein content.

As a consequence of this lack of uniformity in both catabolism and anabolism it follows that dietary alterations are accompanied by a considerable degree of internal rearrangement of the body protein. Table IV shows the changes in the distribution of total protein among various parts during fasting and refeeding with a 70 per cent casein diet.

These figures reveal that the direction of change in the internal organs is contrary to that in the carcass (almost wholly muscle, skin, and skeleton). During fasting there is a progressive decrease in the protein allocated to the organs, while the proportion of the total protein within the carcass increases. When casein

is given, the reverse change occurs, the organs gain while the carcass loses. In other words, so long as catabolic processes prevail,

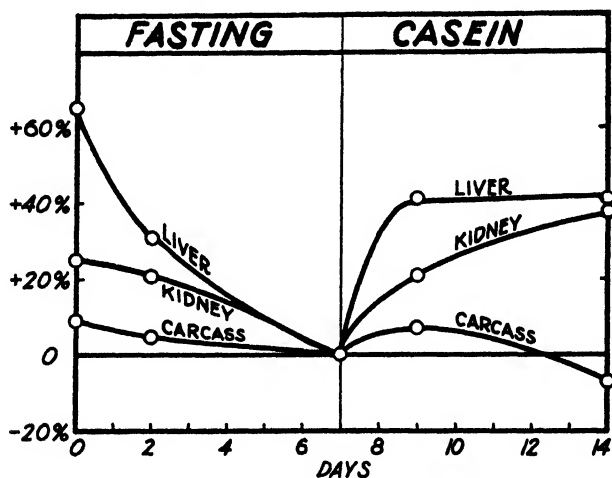


FIG. 1

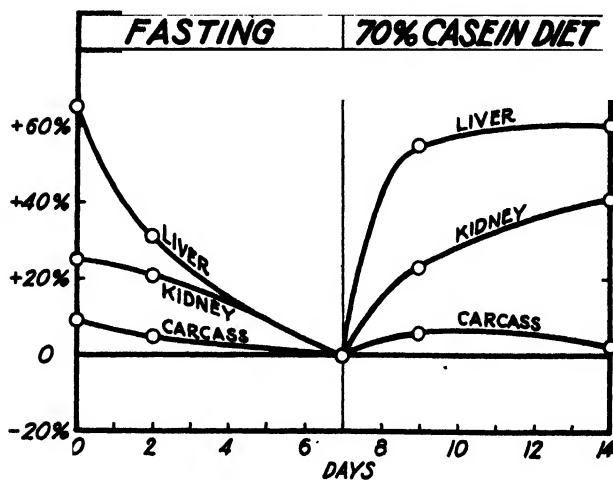


FIG. 2

the internal organs lose in relation to the peripheral supporting and motor structures, but when anabolism is in the ascendent, the central organs gain relatively to the rest of the body.

The relative changes in the protein content of the kidney, liver, and carcass are shown in Figs. 1 to 3. Here the content of each organ at the termination of the period of fasting or of no protein diet is taken as the point of reference and the curves are the percentage changes relative to these quantities.

We interpret the decrease in the kidney protein as a result of atrophy from disuse and the increase when casein is given as due to work hypertrophy. This view is supported by the fact that the decrease, both absolute and relative, is less on a calorically adequate diet than when no food is given. The quantity of

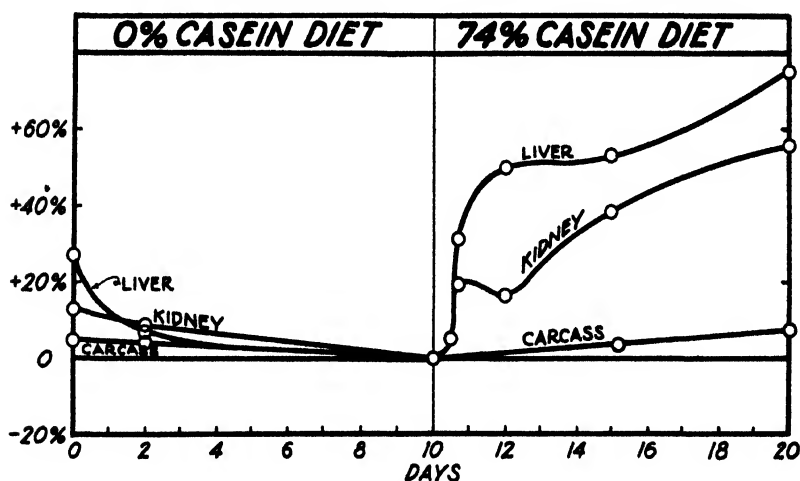


FIG. 3

nitrogen excreted is known to be less under the former than under the latter condition. Our opinion that the increase after casein feeding is due to work requires for validation a demonstration that the degree of increase varies as some measure of renal work, but in these experiments it was not feasible to make such measurements nor even possible to obtain a sufficiently reliable measure of protein consumption.

If atrophy from disuse and work hypertrophy be accepted as the most plausible account of the changes in the kidney, then certainly the different form of the curves for the liver necessitates some other explanation. The precipitate fall when protein is

withdrawn, and, when casein is given, the rapid rise to a point which quickly approaches the maximum inevitably suggest the depletion and subsequent reconstitution of a store of protein. Nevertheless work at present under way shows that this plateau effect is not always obtained when protein is fed after protein depletion. Also an attempt to prove the hypothesis of protein storage in the liver failed. It was based on the idea that nucleoprotein is the only body protein known to contain phosphorus as an integral constituent of its structure, and on the supposition

TABLE V

Relation between Total Phosphorus and Total Protein Content of Liver and Kidney

	Fed	2 day fast	7 day fast	+ 2 days casein	+ 7 days casein	+ 2 days 70 per cent casein	+ 7 days 70 per cent casein
Liver							
Total P (nuclear), mg.....	9.4	8.5	7.1	8.7	7.8	11.0	12.6
Total protein, gm...	1.5	1.2	0.9	1.3	1.3	1.4	1.5
P:protein ratio.....	6.2	7.0	7.9	6.7	6.0	7.7	7.7
Per cent change from 7 day fasted ratio.	-22	-12	0	-15	-24	-3	-2
Kidney							
Total P (nuclear), mg.....	1.2	1.2	1.1	1.2	1.3	1.4	1.7
Total protein, gm...	0.22	0.21	0.17	0.21	0.24	0.21	0.25
P:protein ratio.....	5.5	6.5	6.7	5.6	5.5	6.7	7.2
Per cent change from 7 day fasted ratio.	-17	-3	0	-17	-19	+0	+7

that the phosphorus content of liver protein from which inorganic and lipid phosphorus had been removed should be a measure of its nucleic acid content. Now, if the stored protein was made up solely of phosphorus-free amino acids, its going and coming should be mirrored inversely in changes in the relation between the phosphorus and protein content, in the alterations of this chemical nucleocytoplasmic ratio. Accordingly, we measured the phosphorus in our liver protein preparations. The whole sample, usually about 1 gm. of protein, with its capsule and cotton-wool,

was wet-ashed and the phosphorus determined by Embden's gravimetric method (3). There was excellent agreement in analyses on different preparations obtained under the same dietary conditions. As a contrast to the liver we also measured the phosphorus content of kidney protein preparations, for we supposed that in atrophy and hypertrophy the decrease and increase in both the nucleus and cytoplasm of the cells should be uniform in contradistinction to that waning and waxing of the cytoplasm with constancy of the nucleus to be anticipated under a depletion and reconstitution of a store of protein in the liver. The results are given in Table V.

Although we do not attempt to interpret them, these phosphorus determinations are given here because long ago Kossel (4) and others after him (5) reported that the P:N₂ ratio in the liver rose on fasting and fell when protein was fed and cite this fact as evidence of protein storage. Their experiments were not numerous and they were satisfied with a demonstration of a relative increase and decrease of phosphorus concentration without demonstrating a simple inverse relation between total phosphorus and total protein content. But Table V shows that the nuclear phosphorus does not remain constant as the liver protein decreases on fasting and increases on casein refeeding and so the actual relation is one between too simultaneously changing quantities.

In the end the only entirely satisfactory evidence of a process of protein storage in the liver analogous to the storage of carbohydrate remains the isolation of a definitely characterized protein from the livers of protein-fed animals and the demonstration that this protein disappears on fasting. It is just this evidence which is negated by Luck's careful work (6). This question is an old one but we have refrained from any reference to its history, partly because we come to it with new methods, but mainly because we are well aware that our observations fall only within the first chapter of a story that has not yet been told.

SUMMARY

1. Each organ and tissue has its own individual characteristics with respect to the degree and rate of the rebuilding of protein when casein is given after a period of protein loss.

2. While new protein is being formed after casein administration, the proportion of the total body protein found in the internal organs increases relatively to the proportion in the muscle, skin, and skeleton. During a fast, while protein is being disintegrated, a shift in the reverse direction takes place.

3. In the carcass and heart the changes in the quantity of protein after casein feeding are relatively small. They are large in the kidney and liver. In the kidney the rate of increase is gradual and steady, in the liver it is at first rapid but soon becomes slow.

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HETEROGENEOUS EQUILIBRIUM OF PROTEIN SOLUTIONS

I. ACTIVITY COEFFICIENTS AND MEMBRANE EQUILIBRIUM IN MIXTURES OF GELATIN AND SALTS

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The present state of our knowledge concerning the interaction of isoelectric proteins and inorganic salts in solution has been developed to a large extent by the experimental study of two types of heterogeneous equilibrium, the solubility equilibrium of proteins and their two phase membrane equilibrium. The study of these two types of equilibrium has led to two distinct types of formulation. Loeb (13) and Sørensen (21) applied to membrane equilibrium the equations that had earlier been derived theoretically by Donnan (7), and on the basis of the ideal solution laws related the observed effects, osmotic pressure, membrane potential, and the unequivalent distribution of diffusible ions, to the chemical combination of proteins with acids and bases. This type of chemical reaction has been thoroughly studied by electrometric titrations, and has been invariably found to obey the mass law. It is fully established that in the presence of acid or base the proteins are indiffusible ions.

In a similar manner the application of the Donnan equations to the explanation of membrane phenomena in mixtures of isoelectric protein and inorganic salts has led to the conclusion by Northrop and Kunitz ((18) p. 481) that proteins react with salts to form indiffusible ions. It must be emphasized, however, that in contrast with the reaction of acids and bases with ampholytes, there is relatively little evidence that the formation of the assumed complex ions obeys the mass law.

The second type of heterogeneous equilibrium that has been

extensively studied is the protein solubility. The interaction as determined by this method has been formulated along quite different lines. Cohn and Prentiss (6) expressed it in an equation relating the activity coefficient of the protein to the ionic strength. Formulated in this way it has since been found that the interaction can often be expressed by empirical equations which are somewhat analogous in form to the Debye-Hückel equation for the interaction of ordinary ions. In general this type of study has given little support to the idea of chemical combination of inorganic salts with proteins. Neither does it readily permit the assumption that the ideal solution laws are generally applicable.

Another quite different approach to the problem of heterogeneous equilibrium is the purely thermodynamic one that involves no assumption as to the mechanism of the interaction of the components. Adair (1-3) has stated the equations of membrane equilibrium in such a manner. In his equations the properties of the system are regarded as functions of the chemical potentials of the components, and one can determine from the membrane equilibrium the effect of varying protein concentration on the chemical potential of the protein at *constant chemical potentials of the other components*. Adair's treatment does not readily permit the expression of the activity coefficients as functions of the concentrations of the components.

This limitation can be avoided by taking the concentrations rather than the potentials of the components as independent variables and by the introduction of additional thermodynamic relations between the chemical potentials. Furthermore, if the activity coefficient of one of the components can be expressed in terms of the protein concentration and the salt concentration, the various types of equilibrium can be correlated. It is the purpose of this paper to develop equations describing the equilibria, and to illustrate their application to the study of activity coefficients and membrane phenomena in mixtures of gelatin and various salts.

Theoretical

Let us assume that we are given a solution containing three components, solvent (Component 1), non-electrolyte or isoelectric ampholyte (Component 2) at the molality m_2 , and inorganic salt (Component 3) at the molality m_3 . We assume further that the

interaction between Components 2 and 3 can be expressed at various values of m_2 and m_3 in terms of γ_2 and γ_3 , the activity coefficients of Components 2 and 3, and that these are continuous functions of the molalities. This is equivalent to the assumption that we know at any composition the molal free energy change of transferring either component from an isomolal two component reference solution to the mixture.

$$\Delta F_2 = RT \ln \gamma_2 / \gamma_2^0 \quad (1-a)$$

$$\Delta F_3 = \nu RT \ln \gamma_3 / \gamma_3^0 \quad (1-b)$$

\bar{F}_2 and \bar{F}_3 are chemical potentials, R is the gas constant, T the absolute temperature, γ_2^0 and γ_3^0 the activity coefficients of the reference solutions, and ν denotes the number of gm. ions formed by the dissociation of 1 gm. molecule of strong electrolyte (Component 3). The "activity coefficient of the electrolyte" will be understood to signify the mean activity coefficient of the ions. As defined in Equation 1-b, its application involves no *a priori* assumption regarding the physical or chemical mechanism of the interaction. This is true also of Equation 1-a and of the subsequent thermodynamic derivations, which depend only on energy relations.

Equations 1-a and 1-b are never independent, for the chemical potentials must satisfy differential equations of the form

$$\begin{array}{ccc} \delta \bar{F}_2 & \delta \bar{F}_3 & \delta^2 F \\ \delta m_3 & \delta m_2 & \delta m_2 \delta m_3 \end{array} \quad (2)$$

where F is the free energy.

It follows from Equations 1 and 2 that if either $\ln \gamma_2 / \gamma_2^0$ or $\ln \gamma_3 / \gamma_3^0$ is a known continuous function of m_2 and m_3 , the other function can also be evaluated in terms of the same variables (4, 9). These relations can be stated in the form

$$\frac{\delta \ln \gamma_2}{\delta m_3} = \nu \frac{\delta \ln \gamma_3}{\delta m_2} \quad (3)$$

$$\ln \gamma_2 / \gamma_2^0 = \nu \int_0^{m_3} \frac{\delta \ln \gamma_3}{\delta m_2} dm_3 \quad (3-a)$$

$$\nu \ln \gamma_3 / \gamma_3^0 = \int_0^{m_2} \frac{\delta \ln \gamma_2}{\delta m_3} dm_2 \quad (3-b)$$

The activity coefficients of the reference solutions enter Equations 3-a and 3-b as integration constants.

Let it now be required to determine the osmotic effects of any interaction in terms of either γ_2 or γ_3 and the integration constants that characterize the reference systems. First it will be convenient to examine the relation between the activity coefficient of the solute and the freezing point of two component systems.

Lewis and Randall (12) express this relation at constant temperature in the form

$$-\ln \gamma = j + \int (j/m) dm \quad (4)$$

where γ is the activity coefficient of the solute and $j = 1 - (\theta/\nu m \lambda)$, θ denoting the freezing point depression and λ the ideal molal freezing point depression. The ratio $\theta/\nu m \lambda$ can be identified with the osmotic coefficient φ of Bjerrum.

Equation 4 then becomes

$$-\ln \gamma = (1 - \varphi) + \int_0^m \frac{(1 - \varphi)}{m} dm \quad (4-a)$$

Since we are interested in expressing φ in terms of $\ln \gamma$, it is convenient to transform this expression. Writing it in differential form, we obtain

$$-m d \ln \gamma = m d (1 - \varphi) + (1 - \varphi) dm$$

or
$$d[m(1 - \varphi)] = -d(m \ln \gamma) + \ln \gamma dm$$

Integrating from $m = 0$, we obtain

$$m(1 - \varphi) = -m \ln \gamma + \int_0^m \ln \gamma dm \quad (4-b)$$

This equation holds for the reference systems of both Components 2 and 3. To fit our notation γ becomes γ_2^0 or γ_3^0 , m becomes m_2 or m_3 , and φ will be written φ_2 or φ_3 .

It is now necessary to derive a relation between φ_{23} , the osmotic coefficient, and the activity coefficients and molalities of Components 2 and 3 in the mixed solution. This can be effected by application of the Gibbs-Duhem equation.

$$m_1 dF_1 = -m_2 dF_2 - m_3 dF_3 \quad (5)$$

Integration by parts and introduction of the relation between φ_{23} and \bar{F}_1 leads to the expression

$$(1 - \varphi_{23})(m_2 + \nu m_3) = -m_2 \ln \gamma_2 - \nu m_3 \ln \gamma_3 \\ + \int_0^{m_2} \ln \gamma_2 dm_2 + \nu \int_0^{m_3} \ln \gamma_3 dm_3 \quad (6)$$

When m_2 or m_3 becomes 0 this equation reduces to Equation 4-b. Referring the interaction to the isomolal reference solutions, we find after combining Equation 6 and the two possible forms of Equation 4-b

$$\varphi_{23}(m_2 + \nu m_3) - \varphi_2 m_2 - \nu \varphi_3 m_3 \\ = m_2 \ln \gamma_2/\gamma_2^0 + \nu m_3 \ln \gamma_3/\gamma_3^0 - \int_0^{m_2} (\ln \gamma_2/\gamma_2^0) dm_2 \quad (7)$$

Applying Equation 3-a and multiplying by RT , we obtain

$$P_{23} - P_2 - P_3 = RT \left(\nu m_3 \ln \gamma_3/\gamma_3^0 \right. \\ \left. + m_2 \int_0^{m_3} \nu \frac{\delta \ln \gamma_3}{\delta m_2} dm_3 - \int_0^{m_2} \int_0^{m_3} \nu \frac{\delta \ln \gamma_3}{\delta m_2} dm_2 dm_3 \right) \quad (8-a)$$

where P_{23} , P_2 , and P_3 denote the osmotic pressure of the three component system and the two reference solutions. An equation very similar to Equation 8-a has been applied to the study of freezing point effects in mixtures of amino acids and salts (9).

The relation can be written also in the entirely equivalent form

$$P_{23} - P_2 - P_3 = RT \left(m_2 \ln \gamma_2/\gamma_2^0 \right. \\ \left. + m_3 \int_0^{m_2} \frac{\delta \ln \gamma_2}{\delta m_3} dm_2 - \int_0^{m_2} \int_0^{m_3} \frac{\delta \ln \gamma_2}{\delta m_3} dm_2 dm_3 \right) \quad (8-b)$$

These expressions can be evaluated if either of the activity coefficients is a known continuous function of m_2 and m_3 .

The pressure terms in the above equations refer to osmotic pressures within membranes permeable to water and impermeable to both of the dissolved components. A system more typical in

practice is that in which the membrane is impermeable to only one of the components, as in the scheme:

Inner solution	Outer solution
Isoelectric ampholyte, or non-electrolyte, m_2	
Salt, m_3	Salt, m'_3
Water	Water

In order to derive the properties of such a system it is necessary to consider, in addition to the one phase interaction of the components, the distribution of the salt between the two phases. The outer solution contains only salt and solvent, and the molality of the former will be denoted as m'_3 . In general $m_3 \neq m'_3$, and $P_3 \neq P'_3$.

The observed osmotic pressure, π , in such a system is determined by the relation

$$\pi = P_{23} - P'_3$$

Since we are concerned with the osmotic interaction rather than with the properties of the reference solutions, it is convenient to subtract P_2 from each member, and to add and subtract P_3 , writing this in the form

$$\Delta\pi = \pi - P_2 = (P_{23} - P_2 - P_3) + (P_3 - P'_3) \quad (9)$$

$\Delta\pi$ is the pressure change when the molality of salt is varied from 0 to m_3 . $(P_{23} - P_2 - P_3)$ represents the homogeneous osmotic interaction, and $(P_3 - P'_3)$ represents the phase distribution effect. The former has been related by Equations 8-a and 8-b to the interaction as described by activity coefficients. It is necessary now to relate the latter to the activity coefficients.

According to the definition of the osmotic coefficient, the phase distribution effect is given by the relation

$$P_3 - P'_3 = -\nu RT(\varphi'_3 m'_3 - \varphi_3 m_3) \quad (10)$$

The molality m'_3 is a function of m_2 and m_3 , for according to the laws of heterogeneous equilibrium, $\bar{F}_3 = \bar{F}'_3$, and \bar{F}_3 is a function of m_2 and m_3 . It follows that

$$m_3\gamma_3 = m'_3\gamma'_3 \quad (11)$$

Accordingly, m'_3 can be expressed as a function of m_2 and m_3 , if γ_3/γ'_3 is known in terms of these variables. This ratio differs from γ_3/γ_3^0 by the factor γ_3^0/γ'_3 , which depends on the equilibrium values of m'_3 and m'_3 . But a large difference between m_3 and m'_3 is required to produce a value of γ_3^0/γ'_3 that is greatly different from unity. This presupposes a value of γ_3/γ'_3 that differs from unity by a higher order of magnitude. It is therefore a justifiable first approximation, for small values of m_2 , to consider

$$\gamma_3/\gamma_3^0 = \gamma_3/\gamma'_3 = m'_3/m_3 \quad (12)$$

The validity of this step will later be illustrated by reference to experimental data. From the same considerations it follows also that for small values of m_2 , $\varphi_3 = \varphi'_3$.

Accordingly,

$$P_3 - P'_3 = -\nu RT\varphi_3 m_3 (\gamma_3/\gamma_3^0 - 1) \quad (13)$$

For values of γ_3/γ_3^0 near unity a further approximation is justified

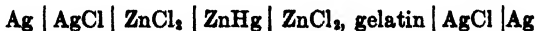
$$P_3 - P'_3 = -\nu RT\varphi_3 m_3 \ln \gamma_3/\gamma_3^0 \quad (14)$$

It is now evident by reference to Equation 8-a that the phase distribution effect can be expected to be of the same order of magnitude but opposite in sign to the homogeneous interaction, and that the net osmotic effect can be expected to be determined to a large extent by the values of the line and surface integrals in Equation 8-a. While the two effects thus tend to balance, an exact balance is to be expected only in limiting cases.

Our next step will be to illustrate the application of the foregoing principles by studying the interaction of gelatin and zinc chloride as expressed by the activity coefficient of the salt.

EXPERIMENTAL

Potentiometric Measurements—Electromotive force determinations have been made by means of cells without liquid junction. The method is identical with that employed in earlier studies on amino acid solutions (9). The cells are of the type



The molality of ZnCl_2 , m_s , is the same in both the reference solution and the protein solution. The cell reaction is a transference of electrolyte from the reference solution to the protein solution, the free energy change being given by Equation 1-b. The E.M.F. is accordingly given by the relation

$$E = -\frac{3RT}{2F} \ln \frac{\gamma_s}{\gamma_s^0} \quad (15)$$

TABLE I
Electromotive Force at 37° of Cell
Ag|AgCl|ZnCl₂|ZnHg|ZnCl₂, Gelatin|AgCl|Ag

Gelatin per kilo water	Salt per kilo water, m_s	E.M.F.	$-\log \gamma_s/\gamma_s^0$
<i>g/l.</i>	<i>mole</i>	<i>volt</i>	
25	0.005	0.0049	0.053
50	0.005	0.0097	0.105
100	0.005	0.0184	0.199
150	0.005	0.0255	0.277
25	0.01	0.0033	0.036
50	0.01	0.0070	0.076
100	0.01	0.0129	0.140
150	0.01	0.0181	0.196
25	0.02	0.0026	0.028
50	0.02	0.0046	0.050
100	0.02	0.0090	0.098
150	0.02	0.0128	0.139
25	0.05	0.0015	0.016
50	0.05	0.0030	0.033
100	0.05	0.0054	0.059
150	0.05	0.0071	0.077
100	0.10	0.0032	0.035
150	0.10	0.0045	0.049

Salt-free isoelectric gelatin was prepared according to the directions of Northrop and Kunitz ((18) p. 477). A 5 per cent solution had a specific conductivity of 2.2×10^{-5} at 25°.

The E.M.F. results at 37° are given in Table I. They show that gelatin decreases the activity of ZnCl_2 , an effect which is diminished as the salt concentration is increased. A very similar result for the interaction of gelatin and zinc ions has been obtained by Northrop and Kunitz (15) by means of cells with liquid junction

and zinc amalgam electrodes. They have expressed their results in terms of combined ions. While chemical combination must be regarded as a possible mechanism by which to account for the interaction, it is more consistent with the thermodynamic treatment outlined earlier in this paper to express the results in a form that implies no particular mechanism.

The interaction of gelatin and zinc chloride can be accurately represented by an empirical equation related thermodynamically to a type of formula which has been applied to the solubility of proteins (8, 20), and which is analogous to the Debye-Hückel equation. In this formula $(-\log \gamma_2/\gamma_2^0)$ is proportional to $m_2^{\frac{1}{2}}$. According to Equation 3 it can be inferred that $(-\log \gamma_3/\gamma_3^0)$

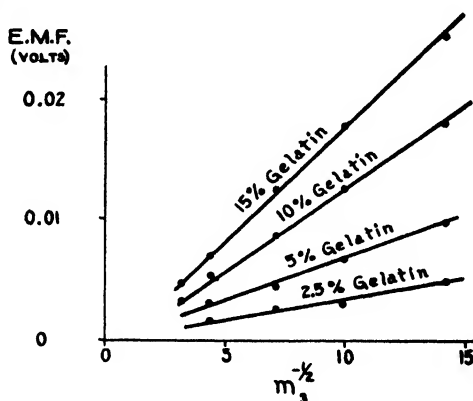


FIG. 1. Influence of gelatin on zinc chloride at 37°

ought to be proportional to $m_3^{-\frac{1}{2}}$. Now within the limits of error of the method, the E.M.F. in mixtures of zinc chloride and gelatin is in fact proportional to the inverse square root of the salt molality. In dilute protein solutions it is approximately proportional to the protein molality. Over the entire range of protein concentration the results can be described by the empirical formula

$$-\log \gamma_3/\gamma_3^0 = (am_3^{-\frac{1}{2}} - b)m_2 - cm_3^{-1}m_2^2 \quad (16)$$

where $a = 10.5$, $b = 5.3$, and $c = 880$. The values for these constants are based on the value 61,500 given by Kunitz, Anson, and Northrop for the molecular weight of gelatin (11). The curves in Fig. 1 are drawn from Equation 16.

From Equations 16 and 3 the effect of zinc chloride on the activity coefficient of gelatin can be deduced. Partial differentiation of Equation 16 with respect to m_2 gives

$$-\frac{\delta \log \gamma_2}{\delta m_2} = (am_3^{-1} - b) - 2cm_3^{-1}m_2$$

According to Equation 3-a, we find

$$\begin{aligned} -\log \gamma_2/\gamma_2^0 &= \int_0^{m_2} \nu [(a - 2cm_2)m_3^{-1} - b] dm_2 \\ &= 2\nu(a - 2cm_2)m_3^{-1} - \nu b m_2 \end{aligned} \quad (17)$$

Thus zinc chloride decreases the activity coefficient of gelatin, and the effect is closely correlated with that of gelatin on the activity coefficient of the salt. The magnitude of this salting-in effect is greater in dilute gelatin solutions than in concentrated ones, a fact which is correlated with the appearance of a higher power of m_2 in Equation 16. Accordingly, at constant salt concentration, the effect of increasing the protein concentration is to increase the ratio γ_2/γ_2^0 .

Now since $2cm_2$ is of a smaller order than a , even at high concentrations of protein, this equation is similar in form to types of empirical equation which have been employed by Cohn and Prentiss (6), by Green (8), and by Palmer (20) to describe the solubilities of various proteins. Expressed in terms of the ionic strength, μ , the relation has usually been given in the form

$$-\log \gamma_2 = 0.5Z_1Z_2\mu^{\frac{1}{2}} - K_s\mu \quad (18)$$

where Z_1Z_2 is a constant, the "apparent valence type" of the protein, so called by analogy with the results of the Debye-Hückel theory of interionic forces. Both Equations 17 and 18 contain a linear salting-out term and a square root salting-in term.

The relations between the various constants are given by

$$0.5Z_1Z_2 = 2\nu a\sqrt{m_3/\mu} \quad (19-a)$$

and

$$K_s = \nu(m_3b/\mu) \quad (19-b)$$

The values derived in this way from zinc chloride-gelatin at 37° are $Z_1Z_2 = 74$ and $K_s = 5.3$. Z_1Z_2 is an empirical constant.

The determination of its physical significance will in all probability depend on the further development of an electrostatic theory of the interaction of ions with multivalent zwitter ions (5, 10).

Determinations of Membrane Equilibrium—Studies of membrane equilibrium in mixtures of isoelectric gelatin and salts by Northrop and Kunitz ((18) p. 481) have shown that in general the total salt molality in the protein phase is greater than that of the outer solution. Regardless of the mechanism of this interaction, this fact indicates according to Equation 12 that gelatin decreases the activity coefficient of the salt.

Fig. 2 illustrates these effects for four different salts, AlCl_3 , LaCl_3 , MgCl_2 , and CuCl_2 . In each case $-\log \gamma_3/\gamma'_3$ is plotted against m_3^{-1} . The values were obtained from Northrop and Kunitz's ((18) p. 481) data by the relation $-\log \gamma_3/\gamma_3^0 = \log m_3/m'_3$. As in the case of the potentiometric results with ZnCl_2 , the relations are linear when plotted in this manner. The results can be expressed by an approximate form of Equation 16, neglecting the term in m_2^2 .

$$-\log \gamma_3/\gamma_3^0 = (am_3^{-1} - b)m_2 \quad (20)$$

For comparison with the potentiometric observations on ZnCl_2 , measurements on the distribution of this salt have been carried out at room temperature, approximately 25° . Osmometers of the type described by Northrop and Kunitz (17) were employed. The protein solution within a collodion membrane was equilibrated with the outer solution. Equilibrium was attained within 24 hours. The equilibrium is described by the data in Table II, which show that gelatin decreases the activity coefficient of ZnCl_2 . The results are in quantitative agreement with those obtained by the potentiometric method. The equilibrium can therefore be characterized by the values of Z_1Z_2 and K , derived from the E.M.F. data.

Table III summarizes the values of Z_1Z_2 and K , for ZnCl_2 and the four salts represented in Fig. 2. The most striking point is that Z_1Z_2 varies widely among the various salts. A discussion of this 6-fold variation of Z_1Z_2 involves extrathermodynamic considerations. This will be deferred until the osmotic relations in the ZnCl_2 system have been analyzed.

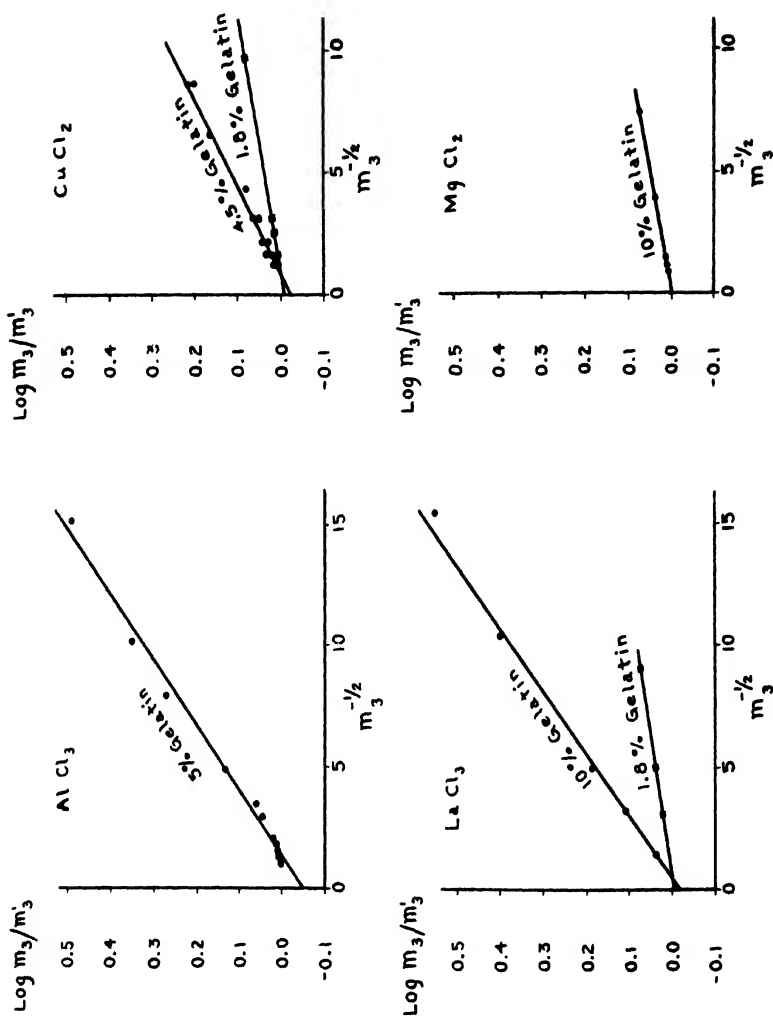


FIG. 2. Influence of gelatin on membrane equilibrium at 37°

Correlation of Osmotic Pressure and Salt Effect

The homogeneous osmotic interaction of gelatin and ZnCl_2 can be calculated by means of Equations 8-a, 16, and 17. According to the former the effect can be analyzed into three terms, the first of which can be obtained by multiplying Equation 16 by the factor $(2.303 \nu RT m_3)$. The second term is the product $(2.303 RT m_2 \log \gamma_2/\gamma_2^0)$, which can be obtained from Equation 17.

TABLE II
Salt Distribution at Approximately 25° (40 Gm. of Gelatin per Kilo of Water)

ZnCl ₂ per kilo water		Log m_2/m_2^0 *	-Log γ_2/γ_2^0 †
Outer solution, m_2	Inner solution, m_2		
mole	mole		
0.0110	0.0123	0.049	0.053
• 0.0304	0.0327	0.032	0.030
0.0408	0.0430	0.022	0.026
0.0649	0.0672	0.015	0.013

* Log γ_2/γ_2^0 estimated by the Debye-Hückel equation is no greater than 0.002. This fact justifies the approximation of Equation 12.

† Calculated, Equation 16.

TABLE III
Values of $Z_1 Z_2$ and K_s in Gelatin Solutions at 37°

	ZnCl ₂	MgCl ₂	LaCl ₃	CuCl ₂	AlCl ₃
$Z_1 Z_2$	74 ± 1	48 ± 0.5	158 ± 3	260 ± 3	306 ± 6
K_s	5.3 ± 1	1.5 ± 0.5	4.1 ± 1.4	50 ± 10	50 ± 20

The third term is the product of $2.303 RT$ and a surface integral which can be evaluated from Equation 17.

$$-\int_0^{m_2} \int_0^{m_3} \frac{\delta \log \gamma_2}{\delta m_2} dm_2 dm_3 = \int_0^{m_2} \nu [(2a - 4cm_2)m_3^{\frac{1}{2}} - bm_3] dm_2 \\ + \nu (2am_3^{\frac{1}{2}} - bm_3)m_2 - 2\nu cm_3^{\frac{1}{2}} m_2^2$$

Accordingly, we find

$$m_2 \int_0^{m_3} \nu \frac{\delta \log \gamma_2}{\delta m_2} dm_3 - \int_0^{m_2} \int_0^{m_3} \nu \frac{\delta \log \gamma_2}{\delta m_2} dm_2 dm_3 = 2\nu cm_3^{\frac{1}{2}} m_2^2 \quad (21)$$

For the osmotic interaction we find

$$P_{23} - P_2 - P_3 = 2.303 \nu RT [-(am_3^{\frac{1}{2}} - bm_3)m_2 + 3cm_3^{\frac{1}{2}}m^2] \quad (22)$$

The difference between the line integral and the surface integral in Equation 21 is independent of the coefficients a and b . It cannot therefore be related to Z_1Z_2 and K_s .

Table IV contains values of $(P_{23} - P_2 - P_3)$ estimated from Equation 22 by applying the values $a = 10.5$, $b = 5.3$, and $c = 880$. These are compared with an estimate based on the same values of a and b , but on the assumption that $c = 0$. As this calculation shows, the effect can be estimated from the values of Z_1Z_2 and K_s only as a first approximation. Only as the limiting

TABLE IV
Osmotic Pressure Effects, Calculated for Mixtures of ZnCl₂ and 10 Per Cent Gelatin at 37° (Mm. of Hg)

m_3 , mole salt per kilo water	$-(P_{23}-P_2-P_3)_{c=0}$	$-(P_{23}-P_2-P_3)$	P_2	P'_3	$\Delta\pi$	$\Delta\pi^*$
0.005	155	90	274	172	+12	
0.01	217	124	536	393	+19	
0.02	299	167	1048	851	+30	+18
0.05	451	243	2575	2290	+42	
0.10	603	367	5138	4740	+31	
0.20						+15
0.50						+10

* Observed, Northrop and Kunitz (15).

case of $c = 0$ is approached in actual systems can one expect to estimate accurately the osmotic effect from the constants that characterize the solubility of proteins.¹

Table IV contains also values of P_3 and P'_3 estimated according to Equations 10, 12, and 16, by the use of osmotic coefficients of ZnCl₂ obtained from the freezing point data in the International Critical Tables. $\Delta\pi$ is then obtained from the homogeneous interaction and the phase distribution effect. The calculated

¹ It should be pointed out that the osmotic calculations are independent of the value taken for the molecular weight of the protein. The values a , b , and c depend on that value, but the products am_2 , bm_2 , and cm_2^2 are independent of it.

values of $\Delta\pi$ are compared with the observations of Northrop and Kunitz (15). In 10 per cent gelatin solutions, ZnCl_2 increases the observed osmotic pressure, which passes through a maximum. This effect corresponds as a first approximation to the theoretical curve estimated from the electrometric data. The discrepancy may be attributed to experimental error in the estimation of c . This occurs in a relatively unimportant higher order term in the equations for the activity coefficients, but in the two phase osmotic effect it is the decisive term, as the calculations in Table IV illustrate. Since it is a relatively unimportant term in Equation 16, it is difficult to estimate with great accuracy, a fact which may be assumed to account for a large part of the error in the osmotic calculation.

It can therefore be concluded that the osmotic effect of ZnCl_2 on gelatin is largely dependent on a term in m_2^2 , which occurs in the equation describing the activity coefficient of the salt. It is to a less extent dependent on linear terms in m_2 , although the latter are the important ones in determining the activity coefficients of the components.

This conception of the problem is entirely consistent with the results of Adair's treatment. From his observations on membrane equilibrium in mixtures of dilute salts and hemoglobin Adair (3) has arrived at values greater than unity for f_p , the activity coefficient of the protein. Now f_p is a function defined differently than γ , the activity coefficient of Lewis and Randall, which is to be identified with that determined by the solubility method. In hemoglobin solutions γ_2 is much less than unity at low salt concentrations (8). Adair relates f_p to γ_2 by the definition

$$\gamma_2 = f_0 f_p$$

where f_0 is a function of the salt concentration. It remains unspecified in Adair's treatment as does γ_2 . But in both treatments the increased osmotic pressure of the protein is related to that part of the protein activity coefficient which varies with the protein concentration. This, as has been pointed out in connection with Equation 17, is correlated with the non-linear terms in m_2 which may occur in the equation relating γ_2 and m_2 .

DISCUSSION

As emphasized in the theoretical section, the application of activity coefficients as thermodynamic functions involves no *a priori* assumption regarding the interaction that is observed. Questions of mechanism arise only when one seeks to account for the form of the empirical equations, the magnitude of such constants as those given in Table III, and the relation of these data to the general class of protein-salt effects.

As has been pointed out, Equation 18 approximately characterizes the interaction of several different proteins with many different salts. The salts that were employed in the solubility studies to which the equation was applied were invariably those of alkali or alkaline earth metals. Within this limitation Z_1Z_2 , the apparent valence type, has been found to be constant for any given protein and independent of the nature of the salt. This fact together with the analogy between Equation 18 and the Debye-Hückel formula is consistent with the hypothesis that the interaction is explicable as the electrostatic effect of ions on zwitter ions. This point of view receives additional support from the theoretical work of Kirkwood (10) and experimental work on amino acid solutions (5).

It has been pointed out in the case of the gelatin systems characterized in Table III that Z_1Z_2 varies widely among different salts. Moreover, the values are higher than those that have been found to characterize other proteins. They range from 48 to 306, as compared with 4 for hemoglobin (8) to 18 for lactoglobulin (20) and approximately 25 for cdestin (8). Now whatever the physical significance of Z_1Z_2 may be, the results in gelatin solutions are difficult to interpret on the hypothesis that the electrostatic properties of the protein zwitter ions are entirely independent of the nature of the surrounding ions. It must be pointed out in this connection that the cations represented in Table III are those of heavy metals. These constitute a selected group, for the alkali cations examined by Northrop and Kunitz ((18) p. 481) were found to exhibit much smaller effects. This fact suggests small values of Z_1Z_2 for gelatin in the presence of salts of the alkali metals.²

² Because of the low molalities of proteins, $\log \gamma_2/\gamma_1^0$ is usually much nearer 0 than $\log \gamma_2/\gamma_2^0$. Consequently, when Z_1Z_2 is small, it is more difficult to estimate from γ_2 than from γ_1 .

It is a well known fact that in the case of other proteins the effects of the heavy metal cations must be distinguished from those of the alkali and alkaline earth metals. This can be illustrated by reference to the results of Mellanby (14) on serum globulin and those of Osborne and Harris on edestin (19). In both cases Z_1Z_2 , as estimated by Green (8) from their data, is constant for the alkali cations, but the effects of certain heavy metal cations must be distinguished quantitatively if not qualitatively from that of the alkali cations.

It is thus evident that the hypothesis of electrostatic interaction in an unmodified form cannot be extended to the case of all inorganic salts. But the fact that even in the case of heavy metal cations the form of the Debye-Hückel equation is retained suggests that as a working hypothesis it is unnecessary to postulate any factor of a qualitatively different kind, such as chemical combination, at least in gelatin solutions. If dissociable chemical complexes were formed between gelatin and inorganic salts, the mass law ought to be applicable to the salting-in effect. The square root law cannot be derived from the mass law for any simple type of reaction. Perhaps by postulating a sufficiently complex chemical mechanism one might derive a square root equation. But even in that case the salting-out term would not be accounted for. An alternative to some such modification of the electrostatic hypothesis for the case of heavy metal cations would require the assumption that the variability of Z_1Z_2 corresponds to some variation in the electric properties of the protein zwitter ions in the neighborhood of these ions. This hypothesis is suggested as the simplest one to account for the thermodynamic properties of gelatin and salts. It is consistent with the experimental facts, and it retains the generality of a formulation based on the hypothesis of electrostatic forces between ions and zwitter ions.

I wish to thank Professor W. C. Stadie for his interest in this work.

SUMMARY

1. Thermodynamic equations have been developed which correlate the osmotic properties of three component systems with the activity coefficients of the dissolved components.

2. The effect of gelatin on the activity coefficient of zinc chloride has been determined electrometrically.

3. The effect of zinc chloride on the activity coefficient of gelatin has been calculated.

4. The salt effect determined electrometrically has been compared with that determined for gelatin by membrane equilibrium and for other proteins by solubility studies.

5. The results are discussed from the point of view of the inter-ionic force theory as applied to the interaction of ions and zwitter ions.

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THE FATE OF DEUTERIUM IN THE MAMMALIAN BODY*

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Investigations in this laboratory (2) of the pharmacological effects of heavy water in various dosages and concentrations have afforded opportunity for a study of the distribution of deuterium in mammalian tissues.

Procedure and Results

As soon as feasible after death each mouse was desiccated in a current of dry air, the moisture being collected in a tube of anhydrous barium perchlorate. The last traces of moisture were removed by drying to constant weight in a vacuum oven at 105°. The water collected was then recovered from the barium perchlorate by slow heating and condensation. It was purified by distillation from potassium permanganate and anhydrous sodium carbonate, followed by distillation from phosphorus pentoxide. When treated in this way, the body water of normal mice had a specific gravity (t/t) of unity, and samples of deuterium oxide mixtures subjected to these distillation processes were found to have the same specific gravity before and after distillation. Thus the method of purification was adequate, and the H_2O-D_2O mixtures were not fractionated by the distillation method employed.

A method similar to that used by Rittenberg and Schoenheimer (3) was employed to determine the proportion of deuterium in the dried tissues. 1 gm. portions of dried tissues were oxidized in an ordinary combustion furnace with cupric oxide as the

* The expenses of this work were largely defrayed by the Research Fund of the Yale University School of Medicine.

† Emerson Fellow, 1934-36.

catalyst and lead peroxide to absorb the oxides of sulfur and nitrogen. The commercial oxygen used was freed from any traces of hydrogen by purification in a separate combustion furnace. The water of combustion was collected in a tube of barium perchlorate and purified in the same manner as the body water.

To effect a partial separation of the fat from the rest of the tissues some of the dried mice were placed in Soxhlet extractors with alundum thimbles and extracted with anhydrous ether. A mixture of alcohol and ether, a more efficient fat solvent, was avoided, as the hydrogen of the alcoholic group would probably be exchanged for the deuterium. The residue from this extrac-

TABLE I
Mice Receiving Single Injections of Heavy Water

Mouse No.	Specific gravity		100 × $\frac{\text{Tissue D}}{\text{Body water D}}$
	Body water	Tissue water	
S-4	1.0065	1.0008	12.3
S-2	1.0064	1.0009	14.1
S-5	1.0069	1.0010	14.5
S-8	1.0072	1.0014	19.4
SC-1	1.0073	1.0010	13.7
SC-2	1.0078	1.0013	16.7
S-169	1.0099	1.0018	18.2
Mean.....			15.6
Standard deviation.....			2.6
Probable error of mean.....			0.66

tion consists of the inorganic material and the nitrogen-rich substance, which is mostly protein.

The proportion of deuterium in all the samples of purified water was calculated from the specific gravity, determined by the falling drop method of Barbour and Hamilton (1). In calculating percentages of deuterium oxide in mixtures, the density ($t\ 25^\circ / t\ 25^\circ$) of pure heavy water was taken to be 1.1074 (5).

To determine the per cent of tissue hydrogen that would be exchanged readily for deuterium each of seven mice was given a single dose of heavy water (three subcutaneously and four by stomach tube) and killed 1 hour later. The densities of the body

water and of the water from the combustion of the tissues were determined. As shown by Table I, almost one-sixth of the tissue hydrogen is in a form readily exchangeable for deuterium.

TABLE II
Mice Receiving Repeated Doses of Heavy Water

Mouse No.	Specific gravity		100 × $\frac{\text{Tissue D}}{\text{Body water D}}$
	Body water	Tissue water	
L-76	1.0097	1.0023	23.7
M-83	1.0100	1.0027	27.0
M-82	1.0103	1.0026	25.3
M-87	1.0112	1.0025	22.3
K-66	1.0113	1.0023	20.3
K-65	1.0126	1.0022	17.4
K-56	1.0126	1.0029	23.0
K-55	1.0160	1.0032	20.0
K-59	1.0172	1.0043	25.0
B-14	1.0282	1.0038	13.5
H-43	1.0292	1.0076	25.9
J-54	1.0298	1.0063	21.1
J-51	1.0309	1.0044	14.2
H-48	1.0331	1.0070	21.1
L-71	1.0331	1.0056	16.1
H-44	1.0335	1.0079	23.6
H-47	1.0356	1.0078	21.9
M-86	1.0357	1.0068	19.0
F-35	1.0409	1.0072	17.6
B-42	1.0441	1.0081	18.4
O-3	1.0474	1.0080	16.8
K-67	1.0480	1.0044	9.2
M-79	1.0537	1.0086	16.0
E-31	1.0541	1.0059	10.9
D-24	1.0561	1.0088	15.6
Mean.....			18.5
Standard deviation.....			4.8
Probable error of mean.....			0.65

Similar analyses (Table II) were made of the mice used in metabolism studies (2) previously mentioned, concerned with the long continued administration of deuterium oxide. The average proportion of tissue water deuterium to that of body water in

this group of animals is significantly greater than in the animals receiving a single dose; this suggests that some of the deuterium is in non-labile positions. Obviously, the amount of deuterium fixed in stable positions will depend not only upon the total amount of heavy water given, but also upon the time an appreciable deuterium concentration is maintained in the body water.

The variation in the proportion of tissue water to body water in the above two groups was marked. Differences in body composition which might account for this were considered. The largest variation is probably the proportion of fat to protein. In pure fat all the hydrogen atoms are attached directly to carbon by relatively stable linkages. According to Schoenheimer and

TABLE III

Distribution in Vivo of Deuterium between Ether-Extractable and Ether-Non-Extractable Fractions of Mammalian Tissues

Sample No.	Specific gravity			D in ether-insoluble fraction D in ether-soluble fraction
	Body water	Water from ether-insoluble fraction	Water from ether-soluble fraction	
1	1.0056	1.0008	1.0003	2.7
2	1.0074	1.0012	1.0003	4.0
3	1.0101	1.0018	1.0010	1.8
4	1.0195	1.0034	1.0015	2.3
5	1.0305	1.0043	1.0014	3.1
6	1.0450	1.0046	1.0013	3.5

Rittenberg (4) none of these should be replaceable by deuterium, whereas in protein approximately 20 per cent of the hydrogen is in a labile form. A separation of the tissues by ether extraction should therefore yield a fatty fraction poor in deuterium and a nitrogen-rich portion containing more deuterium. To test this hypothesis the dried remainders of about twenty mice with known densities of the body water were combined to make six samples of sufficient size for separate analyses of the ether-soluble and ether-insoluble fractions. The results (Table III) show that much more deuterium is associated with the nitrogen-rich portion than with the lipid fraction; the amounts in the two fractions are in a ratio of approximately 3:1. This ratio is not altered signifi-

cantly by an 8-fold change in the density of the body water to which the tissues were exposed.

The extent to which the appreciable amounts of deuterium in the two fractions might be present in labile linkages was tested by injecting or giving by stomach tube a single dose of heavy water, killing the animal shortly afterward, and analyzing the two fractions. The results of the analyses of three animals are shown in Table IV. Again the amount in the fat fraction is considerably less than that in the residue. The small, but defi-

TABLE IV

Comparison of Distribution of Deuterium in Mice after 1 Hour with Its Distribution after 2 Months

Dose of D ₂ O	Mouse No	Specific gravity			$100 \times \frac{\text{D in ether-insoluble fraction}}{\text{D in body water}}$	$100 \times \frac{\text{D in ether-soluble fraction}}{\text{D in body water}}$	$\frac{\text{D in ether-insoluble fraction}}{\text{D in ether-soluble fraction}}$
		Body water	Water from ether-insoluble fraction	Water from ether-soluble fraction			
Single injection	4	1.0065	1.0009	1.0003	13.8	4.6	3.0
	5	1.0069	1.0007	1.0002	10.2	2.9	3.5
	8	1.0072	1.0008	1.0002	11.1	2.8	4.0
15% for 2 mos.	M-82	1.0103	1.0033	1.0012	32.0	11.7	2.7
	M-83	1.0100	1.0034	1.0014	34.0	14.0	2.4
	M-87	1.0112	1.0034	1.0012	30.4	10.7	2.8

nite, amount of deuterium in the fat fraction may be in several substances, other than pure fat, which are also extracted by the ether.

Long continued exposure to heavy water should create optimal conditions for the fixation of deuterium in non-labile linkages in the tissues. To provide these conditions three mice were permitted to drink 15 per cent heavy water for 2 months. At the end of this time the fatty and the nitrogenous fractions were analyzed. The results (Table IV) show that the ratio of deuterium in the two fractions is not appreciably different from that

in the single injection experiments, but the amounts relative to the body water are higher. This is evidence that the animals were able to store a significant amount of deuterium in non-labile linkages.

SUMMARY

Approximately one-sixth of the tissue hydrogen of mice is in a form readily exchangeable for the deuterium of deuterium oxide.

An ether extract of mouse tissues contains about one-third as much hydrogen readily exchangeable for the deuterium of deuterium oxide as does the remaining nitrogen-rich fraction.

Mice drinking 15 per cent deuterium oxide for 2 months have 3 times the concentration of deuterium (relative to body water deuterium) in the tissues as mice receiving a single injection of heavy water. This is evidence for the fixation of deuterium in mammalian tissues in a stable form.

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COMMENTS ON THE MICROVOLUMETRIC SODIUM METHOD OF BALL AND SADUSK*

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At the time of publication of the microvolumetric sodium method by Ball and Sadusk (1), the present authors had completed a study of a similar method. While the results agreed quite well with those of the above authors, there remained certain points of technique in the Ball and Sadusk method which were definitely inferior to those developed in this laboratory, and the demonstrated range of utility was somewhat less. In order that this excellent method might yield its fullest advantages, it seemed desirable to call attention to some improvements and the advantages to be gained through their use.

Ball and Sadusk followed the technique of Kolthoff and Lingane (2) very closely, using a Jones reductor to reduce the uranium from the hexavalent to the tetravalent state, and standard dichromate solution for its back oxidation. The present authors have found that the reduction was much more conveniently performed in a small flask by adding to the solution of the precipitate about 0.5 ml. of saturated liquid cadmium amalgam, as discussed by Kano (3) and Someya (4). The solution was then shaken for 3 minutes, poured off with washing into the titration vessel, leaving the amalgam behind, aerated for 5 minutes to reoxidize any trivalent uranium, and titrated.

The Jones reductor is always difficult to manipulate with small volumes of solution and almost inevitably leads to excessive dilution of the reduced solution. These difficulties were completely overcome with the liquid amalgam technique.

* Aided by a grant from the Research Board of the University of California.

The titration with standard dichromate solution with barium diphenylaminesulfonate as indicator offers no particular difficulties except that the end-point is not particularly sensitive. The present authors compared the use of dichromate with that of ceric sulfate (5) for the titration. The latter reagent was found to be superior, due chiefly to the exceptionally sharp and distinct end-point which ceric sulfate yields with phenanthroline-ferrous sulfate (6) as indicator. It was found that 0.01 *N* ceric sulfate gave a sharper end-point than 0.025 *N* dichromate—the lowest concentration used by Ball and Sadusk. This advantage makes possible the accurate determination of much smaller amounts of sodium than is possible with dichromate. Thus it was found easily possible to determine amounts of sodium as low as 0.28 mg. with a 10 ml. microburette, and when a capillary burette was substituted, amounts of 0.023 mg. were determined, in both cases with an average error of about 0.5 per cent.

Ball and Sadusk have minimized the effect of temperature on the solubility of the triple acetate, an effect which has been shown by Dobbins and Byrd (7) and Salit (8) to be very great. In our experiments it was found necessary to maintain a temperature during precipitation of not more than 20°, and preferably somewhat lower. It should be emphasized that this effect alone may lead to very serious errors.

While the centrifuge, which was used by Ball and Sadusk for separation of the triple acetate precipitate, is an indispensable piece of equipment for many purposes, its proper function has never been the separation of analytical precipitates. Though it is unfortunately rather widely used for this purpose, it has been demonstrated many times that only the greatest care will prevent loss of precipitate. Also, it is considerably slower than filtration. For separation of the triple acetate precipitate the filters which were used in this investigation were either those described by Kirk and Schmidt (9) or by Kirk and Craig (10), depending on the quantity of the precipitate. No difficulties with the filtration and washing were encountered, and a very few minutes sufficed for the complete operation.

To demonstrate the efficacy of the method as carried out by us, a condensed compilation of data is shown in Table I. Urine, blood serum, and stools were analyzed. In all cases the depro-

teinization and dephosphorization of the sample were carried out by the method of Butler and Tuthill (11), with solid mercuric chloride and solid calcium hydroxide. These operations were simple and highly efficacious as compared with the use of trichloroacetic acid and various dephosphorizing agents tried.

It is interesting that the accuracy, as shown by the results with widely different sample sizes and recovery of added sodium, was greater with the smaller samples than with the larger, and serum yielded better results than urine. The smallest samples analyzed contained considerably less sodium than has heretofore

TABLE I
Sodium Analysis in Biological Materials

Sample	No. of analyses	Range of sodium in sample	Average deviation from mean	Oxidising agent
		mg.	per cent	
NaCl solution.....	45	0.792-0.0524	0.8*	Dichromate
Urine (known amounts of Na added to 18 samples)..	36	3.46 -0.0229	1.4	"
Serum (known amounts of Na added to 29 samples)..	58	4.23 -0.280	0.45	"
Stools (known amounts of Na added to 6 samples)..	12	0.523-0.212	1.5	"
Urine (known amounts of Na added to 10 samples)..	20	0.329-0.0230	0.52	Ceric sulfate
Serum (known amounts of Na added to 10 samples)..	20	0.402-0.280	0.495	" "

* Represents deviation from known amount of sodium in sample.

been determined by a volumetric method, and as little as has ever been reported with colorimetric methods.

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THE RELATION OF LEUCINE, ISOLEUCINE, AND NOR-LEUCINE TO GROWTH*

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(Received for publication, August 10, 1936)

Before the isolation and identification of threonine (α -amino- β -hydroxy-*n*-butyric acid) had actually been accomplished (*cf.* McCoy, Meyer, and Rose (1935-1936), and Meyer and Rose (1936)), several investigations, designed to determine the nutritive importance of certain recognized protein components, were already under way in this laboratory. In these studies, advantage was taken of the fact that excellent growth may be induced in rats by the administration of diets in which the nitrogen is supplied in the form of mixtures of the known amino acids supplemented with crude concentrates of threonine. Under such conditions, the removal from the food of an essential constituent naturally is reflected in the weight changes of the experimental animals.

By the use of this procedure, phenylalanine was shown to be indispensable for life, and incapable of being replaced by tyrosine (Womack and Rose, 1934). In a similar fashion, studies have been made of the physiological rôle of leucine, isoleucine, and norleucine. The details of the methods employed and the results obtained are outlined below.

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The data in this paper were presented in abstract before the American Institute of Nutrition in New York, March 28, 1934 (Womack, M., and Rose, W. C., *J. Nutrition, Proc.*, 7, 10 (1934)).

† The experimental data in this paper are taken from a thesis submitted by Madelyn Womack in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate School of the University of Illinois.

EXPERIMENTAL

The concentrate of threonine was prepared from blood fibrin. The contaminating leucine and norleucine were largely removed

TABLE I
Composition of Amino Acid Mixture

	Casein	Mixture V	
		Active amino acids	As used
	gm.	gm.	gm.
Glycine.....	0.45	0.50	0.50
Alanine.....	1.85	1.90	3.80*
Valine.....	7.93	8.00	16.00*
Leucine.....	9.70†	0	0
Isoleucine.....	?	0	0
Norleucine.....	?	0	0
Proline.....	7.63	8.00	8.00
Hydroxyproline.....	0.23	0.30	0.30
Phenylalanine.....	3.88	3.90	7.80*
Glutamic acid.....	21.77	22.00	22.00
Hydroxyglutamic acid.....	10.50	0	0
Aspartic acid.....	4.10	4.10	4.10
Serine.....	0.50	0.50	1.00*
Tyrosine.....	4.50	6.50	6.50
Cystine.....	?	1.25	1.25
Histidine.....	2.50	2.75	
“ hydrochloride.....			3.40
Arginine.....	3.81	5.25	
“ hydrochloride.....			6.35
Lysine.....	7.62	7.70	
“ dihydrochloride.....			11.55
Tryptophane.....	1.50	2.25	2.25
Sodium bicarbonate.....			12.86
	88.47	74.90	107.66†

* Racemic acids.

† Includes isoleucine.

‡ 1.437 gm. of mixture are equivalent to 1.0 gm. of “effective” amino acids.

in the form of their copper salts. Both of these amino acids yield copper salts which are relatively insoluble in water (Brazier, 1930; Caldwell and Rose, 1934), and hence may be separated from

the readily soluble copper salt of threonine. Unfortunately isoleucine yields a water-soluble copper salt. However, by direct crystallization of the free amino acids, followed by precipitation of an aqueous solution of the monoamino acids with ethyl alcohol, the isoleucine content of the threonine fraction was

TABLE II
*Composition of Diets**

Diet No.....	16	17	18	19	20	21	22	23
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Amino acid Mixture V.....	24.2	22.8	22.8	22.8	21.4	21.4	21.4	20.0
Threonine concentrate.....	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Methionine (<i>dl</i> -).....	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Glucosamine hydrochloride (<i>d</i> -).....	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Sodium bicarbonate.....	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Dextrin.....	18.8	18.2	18.2	18.2	17.6	17.6	17.6	17.0
Sucrose.....	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Salt mixture†.....	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Agar.....	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Lard.....	26.0	26.0	26.0	26.0	26.0	26.0	26.0	26.0
Cod liver oil.....	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Leucine (<i>dl</i> -).....		2.0			2.0	2.0		2.0
Isoleucine (<i>dl</i> -).....			2.0		2.0		2.0	2.0
Norleucine (<i>dl</i> -).....				2.0		2.0	2.0	2.0
	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

* Each diet contained 21 per cent of "effective" amino acids, including glucosamine. The vitamin B factors were supplied in the form of two pills daily, each containing 75 mg. of milk concentrated and 50 mg. of tikitiki extract. We are indebted to Dr. G. C. Supplee of The Dry Milk Company, Inc., for the milk concentrate.

† Osborne and Mendel (1919).

reduced to a sufficiently low level for the purpose of these experiments.

The entire process may be described as follows: 3 kilos of crude commercial fibrin, previously ground and extracted with ether, were hydrolyzed with sulfuric acid in the usual manner (*cf.* Berg and Rose (1929)). After careful precipitation of the sulfuric acid with barium hydroxide, the filtered solution was concentrated *in vacuo* to a volume of 3 liters. On cooling, approximately 1 kilo

of amino acids separated, and were removed by filtration. The filtrate was then extracted seven times with 40 liter portions of butyl alcohol. The extractions were carried out at room temperature in large crocks in which the mixture was violently agitated by two motor-driven stirrers. The first extract, which contained rather large amounts of the leucines, was discarded. The six

TABLE III

Total Changes in Weight and Total Food Intakes of Typical Experimental Animals

Litter No.	Rat No and sex	Duration of experiment	Total change in weight	Total food intake	Supplement
		days	gm.	gm.	
3	2116 ♀	28	-12	48	Leucine
	2117 ♀	28	-10	52	Isoleucine
	2114 ♀	28	-20	38	Norleucine
	2115 ♂	28	-19	53*	"
4	2124 ♀	28	+53	186	Leucine, isoleucine, and norleucine
	2125 ♂	28	+50	195	" " " "
	2126 ♀	28	+61	189	" and isoleucine
	2119 ♂	28	+67	178	" " "
	2120 ♂	28	-21	54	" " norleucine
	2121 ♀	28	-22	53	" " "
	2122 ♀	24†	-19	77*	Isoleucine and norleucine
	2123 ♂	28	-21	57	" " "
	2129 ♂	24	+50	161	Leucine, isoleucine, and norleucine
	2130 ♀	24	+52	165	" " " "
5	2127 ♂	24	+56	159	" and isoleucine
	2128 ♀	24	+52	140	" " "
	2133 ♂	24	-13	59†	" and norleucine
	2134 ♀	24	-17	46	" " "
	2131 ♂	24	-16	54	Isoleucine and norleucine
	2132 ♀	24	-16	46	" " "

* Some food scattered.

† The animal died on the 24th day.

remaining extracts were concentrated *in vacuo* until the dissolved water had been removed. The amino acids which precipitated were combined. They amounted to 230 gm. They were dissolved in 1060 cc. of water, and treated with sufficient absolute alcohol to render the resulting solution 70 per cent by volume. On standing, 30 gm. of amino acids separated and were discarded.

To the alcoholic solution 200 gm. of basic copper carbonate were added, and the whole was concentrated almost to dryness *in vacuo*. The drying was completed in an evaporating dish on a steam cone. The copper salts were then ground to a fine powder, and extracted twice with 800 cc. and 400 cc. of water, respectively. The combined filtrates were treated with hydrogen sulfide, filtered from the copper sulfide, and evaporated to dryness *in vacuo*. The residue weighed 102 gm. Preliminary experiments showed

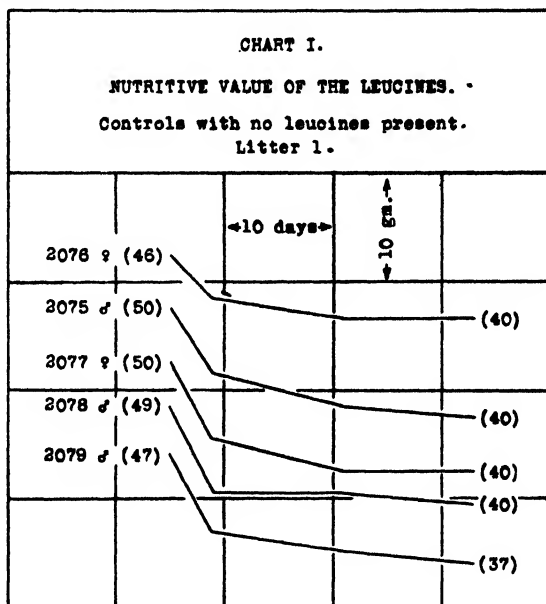


CHART I. The numbers in parentheses denote the initial and final weights of the rats.

that 3 per cent of this material, when incorporated in a threonine-free but otherwise adequate ration, induced normal growth.

Amino acid Mixture V was used in the preparation of the diets. The composition of this mixture is shown in Table I. As will be observed, it was made to imitate the composition of casein, with the exception that the leucines, methionine, and hydroxyglutamic acid were omitted. Hydroxyglutamic acid was not available to us. The leucines and methionine were added directly to the diets in the proportions desired. In accordance with our invariable

practice, each amino acid was recrystallized until a correct analysis was obtained.

Eight diets were employed. Their make-up is shown in Table II. Diet 16 was devoid of the leucines except for such traces as may have been present in the threonine concentrate. Diets 17, 18, and 19 contained *dl*-leucine, *dl*-isoleucine, and *dl*-norleucine,

CHART II.
NUTRITIVE VALUE OF THE LEUCINES.
L - Leucine; I - Isoleucine; N - Norleucine.
Supplements were introduced at the arrows.
Litter 3.

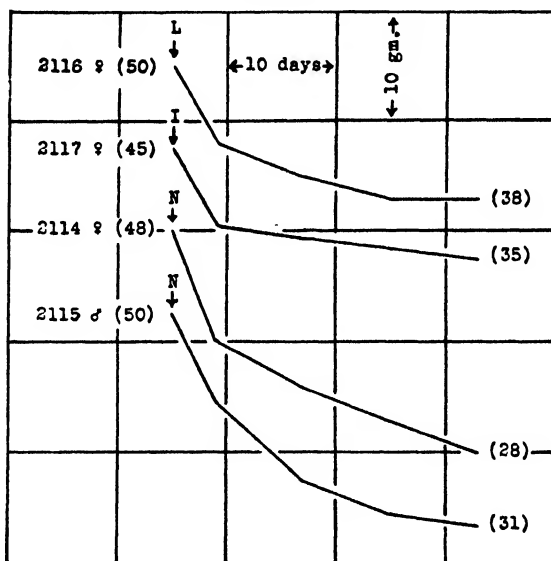


CHART II. The numbers in parentheses denote the initial and final weights of the rats.

respectively. Diets 20, 21, and 22 carried two leucines each, and Diet 23 contained all three of these amino acids. 2 per cent was chosen arbitrarily as the level of each leucine, inasmuch as no information exists as to the amount which is required by the animal organism. Each diet supplied 21 per cent of "effective" amino acids, including glucosamine, and was administered *ad libitum*. The vitamin B factors were furnished in the form of two

pills daily, each containing 75 mg. of milk concentrate and 50 mg. of tikitiki extract.

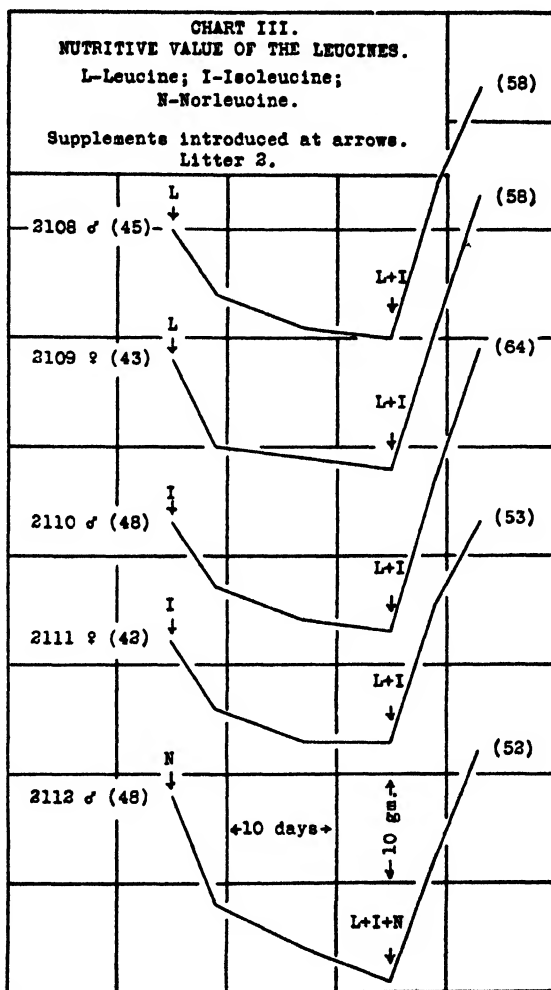


CHART III. The numbers in parentheses denote the initial and final weights of the rats.

Five litters of rats were used, varying in age from 24 to 27 days, and in weight from 42 to 56 gm. The results of the experiments are shown in Charts I to V. In Table III are recorded the total

CHART IV.
NUTRITIVE VALUE OF
THE LEUCINES.

L - Leucine
I - Isoleucine
N - Norleucine

Supplements
were introduced at
the arrows.

Litter 4.

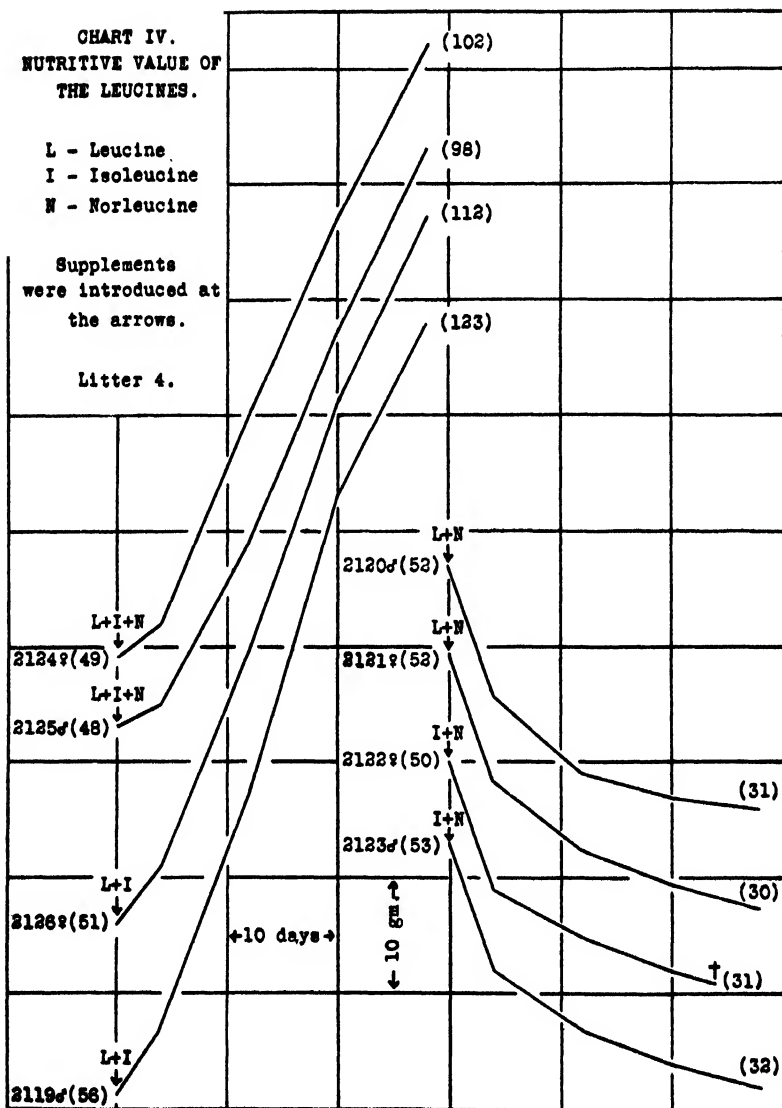


CHART IV. The numbers in parentheses denote the initial and final weights of the rats.

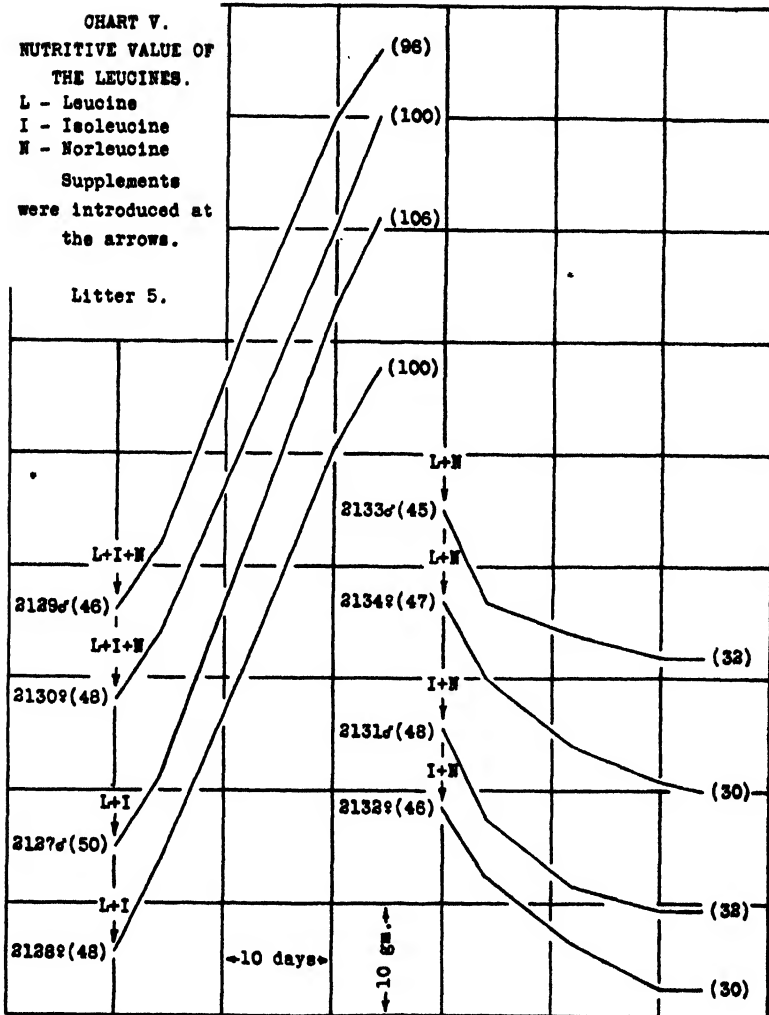


CHART V. The numbers in parentheses denote the initial and final weights of the rats.

changes in body weight and the total food intakes of the animals of three typical litters.¹

As will be seen from Chart I, the absence of leucine, isoleucine, and norleucine from the diet leads to complete nutritive failure. Furthermore, the inclusion of any one of these amino acids, without the others, fails entirely to improve the quality of the food (Charts II and III). On the other hand, when both leucine and isoleucine are present, normal growth ensues (Charts III to V). *Of the three possible combinations of two leucines each, only the one composed of leucine and isoleucine is capable of supporting growth. The results demonstrate conclusively that both of these amino acids are indispensable dietary components.*

The data do not necessarily disprove the possibility that norleucine also may be required for growth. Since no characteristic test for this amino acid is available, it was not possible to determine the amounts which may have been present in the threonine and vitamin concentrates. However, the presumptive evidence is against the idea that norleucine is essential. A comparison of the growth curves shows that when this amino acid was added to the food, the animals almost invariably experienced greater losses in weight, or achieved less rapid gains, than when it was omitted. Apparently, norleucine is not only dispensable, but may actually be detrimental in the proportion employed in these studies. It is quite unlikely that toxic impurities were responsible for the effects, since the compound yielded correct analytical values. In any event, its unexpected behavior will necessitate additional experiments in which the amino acid must be subjected to repeated recrystallization after analytical purity has been attained. Furthermore, the use of crystalline threonine, instead of a concentrate of the latter, will dispose of the present uncertainty as to the rôle of norleucine in growth.

SUMMARY

By the use of diets devoid of proteins, but containing mixtures of highly purified amino acids supplemented with a concentrate of threonine, convincing proof has been obtained for the indispensable

¹ It has not seemed necessary to present all of the data on body weight changes and food intakes. However, those not found in Table III will be furnished to any one desiring them.

nature of leucine and isoleucine. The omission of either from the food leads to a profound nutritive failure.

Apparently, norleucine is not essential for growth. However, final conclusions regarding this amino acid must await the results of additional experiments.

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ENZYMATIC HYDROLYSIS OF LACTALBUMIN*

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Lactalbumin was first separated from whey and identified as an individual protein in 1885 (1). Since then the chief studies with this protein have been concerned with its nutritive value and its relationship to serum albumin, since the latter was thought to be a possible precursor of lactalbumin. Outside of these two phases, lactalbumin has been studied relatively little, and no extensive enzymatic studies have been reported. The most recent one was a comparative study on lactalbumin and casein with unfractionated extracts of the pancreas and intestinal mucosa (2).

With the development of the adsorption methods for the separation of enzymes, it is now possible to study the enzymatic degradation of proteins in successive steps. By such a procedure a fairly complete picture of the structure of some of the protamines has been obtained (3, 4). The structure of the more complex proteins may be partially elucidated by similar studies which would involve an investigation of the successive actions of the individual enzymes and the products in each instance. Only one protein of high molecular weight, ovalbumin, has been so studied (5), and in this case, the second phase has not been completed.

The purpose of this investigation was to subject lactalbumin to the successive actions of pepsin, the enzymes of the pancreas, and finally those of the intestinal mucosa, and thus to ascertain the type of linkage opened and the extent of hydrolysis with each enzyme preparation used. In this manner the foundation

* A preliminary report was presented before the American Society of Biological Chemists at Detroit, April 10-13, 1935 (*Proc. Am. Soc. Biol. Chem.*, 8, lxvi (1935); *J. Biol. Chem.*, 109 (1935)).

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for a more detailed study of a second protein of high molecular weight was laid.

EXPERIMENTAL

Materials—The lactalbumin¹ contained 14.84 per cent nitrogen and 0.66 per cent ash, and at least 7.11 per cent of the nitrogen was in the form of free amino groups. These values and all others reported in this paper have been corrected for moisture and ash. The protein was passed through an 80 mesh screen and then pulverized in a ball mill to an impalpable powder. Suspensions of this material could then be sampled satisfactorily.

A commercial preparation of pepsin (Difco, 1:20,000) was used rather than the crystalline enzyme, since the preparation of crystalline pepsin involves so great a loss of material, which is of significance where large quantities are required. Also, the crystalline and the commercial pepsin have been found to hydrolyze ovalbumin to the same extent (6), and it seemed reasonable to assume that the same would hold in the case of lactalbumin. Incidentally, the studies with which this one is to be compared were not made with crystalline pepsin but with a commercial product.

The enzymes of the pancreas and intestinal mucosa of pigs were separated by selective adsorption methods. The extracts were prepared in the customary manner, as described by Waldschmidt-Leitz (7). The methods of separation and determination of the activity of the various enzymes were those described by Calvery (5). One change was made in the procedure for the test for aminopolypeptidase and dipeptidase; namely, that only one-tenth of the quantity of the substrate and enzyme and 0.02 N solutions of base were used. In one preparation of protaminase, the inactive trypsin was removed by adsorbing it on egg albumin and then precipitating the latter with acetone (8).

Methods

The extent of hydrolysis in all instances was estimated by the Van Slyke amino nitrogen determination. The micromethod was carried out in the customary manner, with a 3 minute reaction period. This was considered adequate for a complete reaction,

¹ The lactalbumin was a commercial preparation obtained from the Harris Laboratories, Tuckahoe, New York.

as the room temperature ranged from 26–28° and the same results were obtained in the 3 and the 30 minute periods with undigested lactalbumin.

For the comparison of the carboxyl and amino groups liberated, the titrimetric methods of Henriques and Sørensen (9), Willstätter and Waldschmidt-Leitz (10), and the "modified Harris," as well as the Van Slyke gasometric method were used. These titrations were carried out on a semimicro scale; *i.e.*, 5 cc. of the hydrolysate which contained approximately 27 mg. of substrate nitrogen were titrated with 0.02 or 0.03 *N* solutions. To stop the hydrolysis, the aliquots for titration were mixed with 10 or 20 cc. of 0.149 *N* alkali in the case of the Sørensen method and with 50 cc. of 95 per cent alcohol in the other titrations.

The titration described by Harris consists of two stages (11). In the first, the alcoholic solution is titrated with 0.1 *N* aqueous sodium hydroxide to the end-point of thymolphthalein; in the second, this mixture is titrated with a similar solution of hydrochloric acid from the alkaline pH to the end-point of methyl red. The titration designated as "modified Harris" refers to the second stage. The modification is the substitution of an 0.02 *N* alcoholic solution of hydrochloric acid for a 0.1 *N* aqueous solution and the replacement of the first stage by the Willstätter method. Thus, at the end of the Willstätter titration methyl red or methyl red-methylene blue was added to the solution and the titration carried to the end-point by the addition of the alcoholic acid solution.

For the Willstätter and modified Harris titration, the standard used for comparison of the end-point consisted of 5 cc. of an aqueous 0.02 *N* solution of pure alanine, 50 cc. of 95 per cent alcohol, the proper indicators, and a quantity of alkali or acid equal to the theoretical plus the blank. This standard was found to be satisfactory with respect to color, as it did not fade for several hours provided the solution was kept in a closed vessel. All titration data have been corrected for the blank obtained by the substitution of 5 cc. of water for the hydrolysate. All the titration methods gave theoretical results with pure alanine.

General Procedures

All the digestions were carried out at 30° in a thermostat. If observations on the early stages of hydrolysis were to be made,

the enzyme and substrate solutions and suspensions were brought to this temperature before they were mixed. Toluene was used as a preservative during all experiments and storage of the hydrolysates.

An enzyme control was carried out with every hydrolysis. In the case of peptic hydrolysis, the control consisted of pepsin and acid in the same concentrations as those mixed with the substrate. In the subsequent experiments, these autolysates of pepsin were treated with enzymes in the same manner as the peptic hydrolysates of lactalbumin.

The data obtained by the Van Slyke method and the titrations represent averages of duplicate or triplicate determinations, except in a few cases in which the material was insufficient for two titrations and in the early stages of peptic hydrolysis where the changes were very rapid. All values have been corrected for the blanks. In the check determinations in the Van Slyke method, the limit of differences allowed was 0.02 cc. of gas, most values being in a closer agreement than this arbitrary limit.

Throughout this paper the extent of hydrolysis, *i.e.* the increase in amino nitrogen as determined by the Van Slyke method and corrected for the autolysis of the enzyme, has been expressed as the per cent of the total nitrogen of the original protein.

DISCUSSION

Peptic Hydrolysis—Two series of experiments were conducted to determine the type of linkage opened by pepsin. In the first series the concentrations of pepsin and lactalbumin were in a 1:9 ratio, and in the second, a 1:4.5 ratio. In the latter case the concentration of the pepsin was 8 mg. per cc. and the protein 36.3 mg. The concentration of the hydrochloric acid in these experiments was 0.175 N or 0.63 per cent, a quantity sufficient to maintain the pH between 1.6 and 2.2 during the entire experimental period. A control consisting of lactalbumin and acid and the usual enzyme control were carried out. In the first series, examinations were made immediately after mixing, at the end of 3, 21, and 23 days, whereas in the second, they were made at shorter intervals, as may be seen in Table I. The results of the first series are not given, since they are similar to those of the second.

The data show a 1:1 liberation of carboxyl and amino groups, which should result if the peptide linkage is broken. The data are concordant within the limits of the experimental error, except for the first three titrations by the Sørensen method. At the end of the titration in these instances the volume of the hydrolysate-formaldehyde-sodium hydroxide mixture was 23 cc. or less. In subsequent titrations, the dilution factor was avoided by increasing the amount of sodium hydroxide necessary to inhibit the digestion.

TABLE I
Digestion of Lactalbumin by Pepsin

The concentrations of lactalbumin and pepsin were 36.3 and 8 mg. per cc., respectively. The increases in titration are expressed in terms of cc. of 0.02 N solution for 5 cc. aliquots, i.e. for 181.5 mg. of lactalbumin. The increases in amino nitrogen (Van Slyke) have been calculated as cc. of 0.02 N solution per 5 cc. of aliquot. All results have been corrected for controls.

Digestion period	Titration			Amino N (Van Slyke)	
	Sørensen	Willstätter	Harris		
hrs.	cc.	cc.	cc.	cc.	per cent total N
1	4.8	6.6	7.1	6.7	7.0
1½				7.2	7.4
1½	5.7	7.4	7.6	7.6	8.0
4	7.7	9.8	9.7	9.9	10.4
8				10.4	10.9
24	13.8	14.8	13.4	14.2	14.9
168	18.6	18.8	17.4	17.2	18.6

The rate of hydrolysis during the early stages is also shown in Table I, at which time the digestion was very rapid. The cleavage effected during the 1st hour expressed as amino nitrogen was equivalent to 7.0 per cent of the total nitrogen, or more than one-third of that which occurred during 7 days, and in 4 hours it was 10.4 per cent, or more than one-half. At the end of 7 days, 18.6 per cent of the total nitrogen had been converted into detectable amino nitrogen.

After the rapid hydrolysis of the first few hours, the rate progressively decreased. The slow but definite cleavage of the later

stages was also demonstrated with eight other peptic hydrolysates, which were observed for periods of 28 to 76 days. In six of these with a pepsin-substrate ratio of 1:4.5, the increase in amino nitrogen by the 21st day was equivalent to 20.5 to 21.9 per cent of the total nitrogen. In four instances, where the experimental period was continued to the 52nd day, the total cleavage ranged between 22.5 and 23.7 per cent. The two most extensively cleaved digests, in which no detectable change was observed between the 66th and 76th days, had 24.1 and 24.7 per cent of the nitrogen converted into the amino form.

It was thought possible that the acid and not the enzyme was responsible for the slow, prolonged hydrolysis. To test this hypothesis, 10 cc. portions of the hydrolysates at various stages of digestion were removed, heated, and maintained at 80-85° for 5 minutes. They were then rapidly cooled to 30°, and the observations were then continued for 11 to 14 days. Heating these acid solutions did not produce any detectable change in the amino nitrogen. The various aliquots represented stages of digestion in which the increase in amino nitrogen prior to this heat inactivation of the pepsin ranged from 7.0 per cent of the total nitrogen to 24.3 per cent. In the lactalbumin-acid control for the experiments described above, the maximal change was equal to but 0.4 per cent of the total nitrogen in the course of 7 days. This value is within the experimental error that occurs when the lactalbumin is not in solution, as is true in this case. These observations indicate that the hydrochloric acid as used in these experiments caused no significant changes and could not have been responsible for the slow hydrolysis observed over long periods of time.

To ascertain the influence of the concentration of pepsin on the extent of hydrolysis, two kinds of experiments were planned. In one kind, all of the pepsin (three different concentrations being used in each series) was added to the substrate at the beginning of the experiment. In the other, more pepsin was added to hydrolysates which had been previously treated with pepsin and allowed to digest until no further increase in amino nitrogen could be detected.

Two series of the first type were carried out with similar results. In these the three concentrations of pepsin used were 8,

16, and 32 mg. per cc., so that the relationships of enzyme to lactalbumin were 1:4.5, 2:4.5, and 4:4.5, respectively. By the 17th day the percentages of hydrolysis in the first series were 19.3, 22.4, and 25.7 for the respective concentrations, and by the 28th day the percentages were 21.7, 23.3, and 26.0, respectively. The results for the second series are shown in Table II.

Six experiments of the second type were carried out. On the 29th day, 8 mg. of pepsin were added for each cc. of an aliquot of Hydrolysate VI. The cleavage effected in the following 29

TABLE II

Hydrolysis of Lactalbumin with Various Concentrations of Pepsin As Shown by Increases in Amino Nitrogen during Digestion

The concentration of lactalbumin was 36.3 mg. per cc.; of pepsin, 8, 16, and 32 mg. per cc. for Hydrolysates VI, VII, and VIII, respectively. All increases in amino nitrogen (Van Slyke) are expressed as per cent of total nitrogen.

Time	Hydrolysate VI	Hydrolysate VII	Hydrolysate VIII
<i>hrs.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
19	20.2	22.4	25.5
21	20.9	22.3	25.5
25	21.9	23.4	26.5
28	21.5	23.3	27.4
29	21.8		
31		23.2	
33	21.6		
34		23.4	26.8
37			28.0
42	22.8	25.0	28.1
53	23.7	24.6	29.0
60	23.9	24.9	28.3

day period was such that the total digestion was equivalent to 25.1 per cent of the total nitrogen, which was practically the same as that of Hydrolysate VII on the 42nd day, in which there was the same amount of pepsin (see Table II). Similarly, a further cleavage in Hydrolysate VII was caused by the addition of 16 mg. of pepsin per cc., which made the total quantity of enzyme used the same as in Hydrolysate VIII. In four cases, the amount of pepsin added to different hydrolysates was equal to 40 mg. for each cc. of the aliquots, making the total quantity

that had been used 48 mg. per cc., and an 8:6 ratio for the enzyme and substrate. The cleavages effected were equivalent to 7.9, 6.8, 7.7, and 6.7 per cent, or a total of 33.5, 32.2, 30.2, and 29.9 per cent, respectively. These data indicate quite clearly that the extent of digestion depends upon the concentration of the enzyme.

The cleavages may also be expressed in terms of the number of linkages broken by acid hydrolysis. The increase in amino nitrogen produced by heating the protein with 20 per cent hydrochloric acid for 32 hours was equivalent to 72.4 per cent of the total nitrogen. On the assumption that this represents complete hydrolysis (100 per cent), the hydrolyses effected by the various concentrations of pepsin would indicate a 29 to 39 per cent cleavage of the peptide linkages.

The extent of the hydrolysis of lactalbumin effected by pepsin is dependent upon the concentration of the enzyme. None of the observations reported here indicates that the maximal cleavage has been obtained as in the case of ovalbumin (5). For that protein where the amount of pepsin was about twice the quantity of substrate, no further digestion occurred after more enzyme was added. The cleavage was equivalent to 25.6 per cent of the total nitrogen or about one-third of the linkages opened by acid hydrolysis. In any experiment where such high concentrations of enzyme are used, the correction for the autolysis of the pepsin is large. Furthermore, this correction for the autolysis of pepsin in any concentration may not be accurate, as it is possible that the changes occurring in the enzyme control and in the digestion mixture may not be identical. The extent of cleavage observed for lactalbumin falls within the limits reported for various other proteins of high molecular weight, 20 to 40 per cent of the total nitrogen (5, 12-15).

The specific interpretation to be placed on the respective titrations depends upon the acceptance or rejection of the zwitterion theory as applied to the products of peptic hydrolysis. According to the classical theory, the Sørensen and the Willstätter titrations measure the carboxyl groups, and the modified Harris the amino groups. But if the free amino and free carboxyl groups form an inner salt or a zwitter ion, the first two methods are essentially back titrations of the $-\text{NH}_3^+$, *i.e.* a conversion to an unionized amino group, and the third is a back titration of the

—COO⁻ group. If one considers that the products of peptic hydrolysis have a zwitter ion structure, then the 1:1 relationship between the Van Slyke data and those of the Sørensen and the Willstätter titrations cannot be interpreted as the liberation of one amino group for each carboxyl group. It may mean that no significant liberation of imino groups, which would be measured by these titrations but not by the Van Slyke method, has occurred, or that the inherent errors of the methods have masked the differences. Therefore, to relate the amino and carboxyl groups liberated, a comparison of the data obtained in the Van Slyke, Sørensen, and Willstätter methods should be made with that of the modified Harris. As the data obtained by all four methods employed showed a 1:1 relationship, the peptide groups only are opened, and neither ester linkages nor those involving the nitrogen of proline have been split. If these other linkages are hydrolyzed, the changes are so small that these methods are not sufficiently sensitive to detect them.

* *Action of Enzymes of Pancreas and Intestinal Mucosa on Peptic Hydrolysates of Lactalbumin*—The hydrolysates used as substrates for this part of the study were those which had a pepsin-lactalbumin ratio of 1:4.5, as described in the first part. At the end of those experiments, the pepsin digests were heated with stirring at 80-85° for 5 minutes in a boiling water bath, cooled immediately, and stored in a refrigerator for 2 to 15 months. As two of the hydrolysates showed a slight increase in amino nitrogen during storage, Van Slyke determinations were made just prior to each subsequent experiment.

The procedure in these experiments was to adjust the hydrogen ion concentration of aliquots of the peptic hydrolysates and the pepsin controls to the required pH, then to add the enzyme preparation, make up to a definite volume, and immediately take samples for the titrations and the Van Slyke amino nitrogen determinations. In the experiments involving aminopolypeptidase and the extract of the intestinal mucosa, the pH of the mixtures was 7.5 and for all others the pH was 8.0.

Representative data of individual experiments and a summary of all the results are given in Table III.

Protaminase—The maximal hydrolysis was equivalent to 9.8 per cent of the total nitrogen. In some instances the digestion

TABLE III

Action of Enzymes of Pancreas and Intestinal Mucosa on Peptic Hydrolysates of Lactalbumin

In Columns 2 to 4 the values of representative experiments are given. In Columns 5 to 7 the total number of experiments in each series, the range of the hydrolyses effected by these enzymes of the pancreas or intestinal mucosa, and the range of the total cleavage are recorded. The same peptic hydrolysate (IX) was used in all experiments for which detailed results have been given except for the protaminase study in which Hydrolysate X was used. All increases in amino nitrogen (Van Slyke) are expressed as per cent of total nitrogen.

Enzymes (1)	Time (2)	Hydrolysis (3)	Total hydrolysis* (4)	No. of experiments (5)	Hydrolysis (6)	Total hydrolysis* (7)
	<i>hrs.</i>	<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>
Protaminase	0		21.3	8	4.3-9.8	28.2-33.9
	24	7.2	28.5			
	48	8.6	29.9			
	68	9.8†	31.1			
Trypsinkinase	0		22.1	3	7.0-8.6	29.2-33.4
	21	5.5	27.6			
	49	5.9	28.0			
	117	7.5	29.7			
Protaminase and trypsin- sinkinase	0		22.1	3	10.0-14.1	35.0-36.4
	21	7.8	29.9			
	48	12.4	34.5			
	96	12.6	34.7			
	168	14.1†	36.2			
Protaminase, trypsin- kinase, and carboxy- polypeptidase	0		22.1	3	17.4-21.9	43.8-44.3
	18	16.0	38.1			
	90	16.2	38.3			
	120	21.9	44.0			
	144	21.9†	44.0			
Aminopolypeptidase	0		21.5	1	15.5-16.4	37.0-37.9
	12	2.2	23.7			
	39	6.9	28.4			
	96	13.6	35.1			
	168	14.5	36.0			
	192	14.5	36.0			
	216	15.5	37.0			
	240	16.4	37.9			
	264	15.5‡	37.0			

* Total hydrolysis is that due to pepsin plus that effected by the enzymes used in this series of experiments.

† Further addition of the enzyme preparations caused no further hydrolysis in 30 hours.

‡ No dipeptidase was detectable in the enzyme control when tested at this point.

TABLE III—*Concluded*

Enzymes (1)	Time (2)	Hydrolysis (3)	Total hydrolysis* (4)	No. of experiments (5)	Hydrolysis (6)	Total hydrolysis* (7)
	<i>hrs.</i>	<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>
Extract of intestinal mucosa	0		21.5	8	28.9-40.5	55.1-62.0
	24	27.3	48.8			
	48	31.8	53.3			
	72	33.9	55.4			
	96	36.2	57.7			
	120	38.6	60.1			
	168	40.3	61.8			
	192	40.5†	62.0			
Extract of pancreas	0		22.1	3	39.8-47.0	66.2-68.3
	144	45.6	67.7			
	192	45.6§	67.7			

§ Further addition of pancreatic extract caused no further hydrolysis in 30 hours. The addition of intestinal extract caused no further hydrolysis in 6 days.

was as low as 4.3 per cent, which may not have been maximal, because the preparation was not very active and the lack of material made it impossible to test for the completeness of the hydrolysis. Considering only the data from those experiments in which the completeness of digestion was established, the amino nitrogen liberated was equivalent to 9 or 10 per cent of the total nitrogen.

Trypsinkinase—With three different peptic hydrolysates, trypsin kinase effected similar degrees of hydrolysis, equivalent to 7.0, 7.9, and 8.6 per cent of the total nitrogen, respectively. These substrates varied in the extent of peptic digestion from 21.3 to 26.4 per cent of the total nitrogen. The latter value was for one of the hydrolysates in which the amino nitrogen had increased during storage so that the total digestion changed from 24.7 per cent of the total nitrogen to 26.4 per cent.

Carboxypolypeptidase—At the time that this experimental work was initiated, pure carboxypolypeptidase had not been isolated. Therefore, the action of this enzyme had to be determined by difference, with protaminase and trypsin kinase in one preparation, and these two enzymes plus carboxypolypeptidase in the other.

The cleavage effected by the combined action of protaminase

and trypsin kinase varied from 10.0 to 14.1 per cent of the total nitrogen, but the total cleavage in the three different hydrolysates was about the same. Therefore, the degree of hydrolysis produced by these enzymes is probably dependent upon the extent of the previous peptic digestion. The sum of the hydrolyses effected by the separate action of protaminase and trypsin kinase is approximately 17 per cent, whereas the simultaneous digestion by these two is 10 to 14 per cent. This would indicate that some of the same linkages are broken by each of these enzymes. In such a case, the calculated values would include the cleavage of some of the groups twice and would of necessity be greater than those observed for the simultaneous action of these two enzymes.

The extent of hydrolysis effected by the mixture of protaminase, trypsin kinase, and carboxypolypeptidase appears to depend upon the degree of the previous digestion, as was the case with the combined action of protaminase and carboxypolypeptidase. The range in values for the digestion produced by this mixture of 3 per cent enzymes was from 17.4 to 21.9 per cent of the total nitrogen, a difference of 4.5 per cent, whereas the difference between the highest and the lowest values for the total digestion (the above hydrolyses plus those previously due to pepsin) was only 0.5 per cent.

The hydrolyses due to carboxypolypeptidase, *i.e.* the difference between the action of the protaminase-trypsin kinase and the protaminase-trypsin kinase-carboxypolypeptidase mixtures, were 7.2, 7.8, and 8.0 per cent for Hydrolysates I, IX, and X, respectively (Table III). It cannot be assumed that this is the cleavage that this peptidase would have effected had the protaminase and the trypsin kinase not been present, since these two enzymes may furnish through their action additional substrate for the carboxypolypeptidase. Furthermore, carboxypolypeptidase might have split these linkages in the absence of the other two enzymes. However, this cleavage by carboxypolypeptidase is maximal under these experimental conditions.

Aminopolypeptidase—Difficulty was experienced in the preparation of aminopolypeptidase free of dipeptidase.² Two such

² The authors wish to express their appreciation to Dr. A. K. Balls for the colloidal iron oxide which was used in the separation of aminopolypeptidase and dipeptidase.

preparations were obtained, but in the first of these, dipeptidase became active during the course of the experiment. Therefore, only the results with the preparation which remained uncontaminated have been reported. In this instance, the cleavage was equivalent to 16 per cent of the total nitrogen, making a total hydrolysis of about 37.5 per cent. Complete digestion may be assumed, since no hydrolysis occurred during the last 48 hours of observation, and the enzyme control was active at the end of 264 hours toward the test substrate, leucylglycylglycine.

Dipeptidases of Intestinal Mucosa—As with carboxypolypeptidase, so here it was assumed that the difference in the digestion caused by the extract of the intestinal mucosa and aminopolypeptidase represents the action of dipeptidase or peptidases which have not been isolated. The absence of trypsin kinase was established, and since tests on many occasions had always shown that protaminase and carboxypolypeptidase were absent when there was no trypsin kinase present, they were not tested for in this instance.

The cleavage effected by this extract may be influenced by the extent of the previous peptic hydrolysis or by the age of the hydrolysate, as the two hydrolysates digested the least in this series of experiments were those which had been most extensively digested by pepsin and had been stored the longest following the heat treatment. In the hydrolysates in which the peptic digestion had been 21 or 22 per cent and storage 2 to 6 months, enzymes in this extract effected a maximal cleavage equivalent to approximately 40 per cent, the total hydrolysis being 61 to 62 per cent. With the two hydrolysates digested most extensively by pepsin and stored 10 to 12 months, the cleavage by the above extract was 28.9 to 29.6 per cent (maximal), giving a total digestion of 55.1 to 56.0 per cent. The significance of these observations is not recognized at present.

The difference between the digestion effected by this mixture of enzymes and by aminopolypeptidase is equivalent to approximately 24 per cent of the total nitrogen. These values cannot be assumed to represent the action of the dipeptidase alone, since the action of aminopolypeptidase and possibly other peptidases may have produced substrates cleavable by dipeptidase.

Combined Action of Enzymes in Pancreatic Extract—With the

three hydrolysates (I, IX, and X), the cleavage produced by the pancreatic extract was equivalent to 39.8, 45.6, and 47.0 per cent of the total nitrogen, respectively, making the total hydrolyses range from 66.2 to 68.3 per cent. These hydrolyses were maximal, as neither the addition of pancreatic extract nor that of the intestinal mucosa caused further digestion.

A comparison of the data obtained from the Willstätter titrations with those for the amino nitrogen as determined by the Van Slyke method shows in this series good agreement. For the majority of experiments, the values obtained by the two methods varied between 0.0 and 0.9 cc. of 0.02 N base per 100 mg. of lactalbumin, and in but nine cases out of thirty-eight were the differences more than 1.0 cc.

The cleavage of the peptic hydrolysates of lactalbumin by trypsin-kinase, although not marked, is significant. This behavior of lactalbumin differs from that of ovalbumin, as with the latter no further digestion was effected by trypsin-kinase following pepsin (5). Had the peptic hydrolysis of lactalbumin been maximal, as it was in the case of the other protein, trypsin-kinase possibly would not have had any action.

One action of protaminase on the protamines is to split off the terminal arginine molecule on the carboxyl end of the chain. If this were the only action in the cases of lactalbumin, the maximal cleavage should have been equivalent to about 1.6 per cent of the total nitrogen, whereas the observed cleavage was 9.8 per cent. Similarly, the action of this enzyme on ovalbumin was greater than could be accounted for by arginine liberation. A study of the products of protaminase action should be made with special attention to the basic amino acids.

It is interesting that under the experimental conditions of this investigation the action of carboxypolypeptidase caused an increase in amino nitrogen equivalent to about 8 per cent of the total nitrogen, that of aminopolypeptidase 16 per cent, and that of dipeptidase 24 per cent; these are different from those cleavages observed with peptic hydrolysates of ovalbumin as substrates (5). The maximal hydrolyses effected by the extract of the pancreas were 6 to 11 per cent greater than those by the extract of the intestinal mucosa. The difference may represent the linkages that are broken by protaminase, trypsin-kinase, or carboxypolypeptidase alone, or by their combined action.

The maximal enzymatic cleavage was equivalent to 94 per cent of the linkages broken by acid hydrolysis. This observation is in agreement with those for numerous proteins subjected to pepsin and unfractionated extracts of the pancreas and intestinal mucosa (16).

The interpretation of the comparison of the amino nitrogen by the Van Slyke method and the Willstätter titration as discussed above depends upon the zwitter ion theory. In view of these considerations, the agreement among the data obtained by these two different methods probably indicates that no significant number of imino groups has been liberated by these enzymes.

SUMMARY

1. During the peptic hydrolysis of lactalbumin, the peptide linkage is assumed to be the principal one broken since amino and carboxyl groups were liberated in equivalent amounts.

2. The rate of hydrolysis was rapid during the first 4 hours and then progressively decreased. A slow digestion, however, continued for 2 months or more, under the experimental conditions of the present study.

3. The hydrochloric acid (0.175 N) used in the maintenance of the pH of the peptic digests of lactalbumin played no significant rôle as a hydrolyzing agent under the experimental conditions employed.

4. The extent of hydrolysis was dependent upon the quantity of pepsin used. Under the experimental conditions of the present study, the number of the peptide linkages broken when the pepsin and lactalbumin were in a 1:4.5 ratio was about 29 per cent of those liberated by acid hydrolysis, or 21 to 22 per cent of the total nitrogen was converted into detectable amino nitrogen. The cleavage could be increased by the use of larger quantities of enzyme.

5. After digestion with pepsin, the hydrolysis effected by the various enzymes of the pancreas, as represented by the increase in amino nitrogen, was equivalent to approximately 10 per cent of the total nitrogen with protaminase, 7 to 9 per cent with trypsin kinase, 10 to 14 per cent with the combined action of these two, 17 to 22 per cent with the combined action of these two plus carboxypolypeptidase, and 40 to 47 per cent with the unfractionated extract.

6. In terms of increase in amino nitrogen, the hydrolysis effected

by aminopolypeptidase obtained from an extract of the intestinal mucosa was equivalent to 16 per cent of the total nitrogen, while with the unfractionated extract this was equivalent to 29 to 40 per cent of the total nitrogen.

7. The maximal enzymatic hydrolysis of the lactalbumin used in this investigation was obtained with pepsin plus the pancreatic extract, and was equivalent to 68 per cent of the total nitrogen or about 94 per cent of the linkages broken by acid hydrolysis.

8. To ascertain the type of linkage broken when the various enzymes in the extracts of the intestinal mucosa and the pancreas acted upon the peptic hydrolysates of lactalbumin, the Willstätter titration as well as the Van Slyke amino nitrogen determination was used. If the zwitter ion theory is accepted, the close agreement of the values obtained by the two methods may be interpreted as indicating that the linkages broken were those in which the nitrogen liberated was in the form of primarily amino groups and not imino groups.

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METAPHOSPHORIC ACID IN THE EXTRACTION AND TITRATION OF VITAMIN C*

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Of the many methods that have been proposed for the direct chemical determination of vitamin C in plant and animal tissues, those based upon the reduction of 2,6-dichlorophenol indophenol have been adopted far more widely than others. The original indophenol procedure proposed by Tillmans and associates (1) a short time before the vitamin was isolated and identified (2) was soon modified in varying degrees in a number of laboratories (3-6). The indophenol method depends upon the fact that vitamin C is the major or only natural tissue component which reduces the dye rapidly in an acid solution (*e.g.* pH 2 to 4).

The present paper deals primarily with the value of having metaphosphoric acid in the acetic acid or trichloroacetic acid used for extracting and titrating the vitamin. In general we have found the procedure outlined by Bessey and King (5) more satisfactory than other methods which have been proposed for tissue analysis, and many other laboratories appear to have had essentially the same experience. The first suggestion of using metaphosphoric acid which came to our attention was in the paper by Fujita and Iwatake (7).

EXPERIMENTAL

Atmospheric Oxidation of Vitamin

It seems reasonably evident that nearly all tissues containing vitamin C also contain one or more enzymes which catalyze its aerobic oxidation to a marked degree as soon as the cellular tissue

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is crushed or severely injured (8-11); hence this factor is of extreme importance in tissue analysis. We have found that it is generally essential to immerse the tissue in a strong acid before it is chopped fine or ground for extraction. Fluids such as urine, milk, and vegetable or fruit juices should be collected directly into an acid, preferably metaphosphoric acid with acetic or trichloroacetic acid, in such quantity that the final concentration is about 2 per cent of the former and 4 to 8 per cent of the latter. In working with animal tissues, such as tumors (12), we have found it advantageous to freeze the tissue in dry ice until it is ready for extraction. It is then pulverized in a glass mortar, covered with the mixed acid (before thawing), then ground, and extracted as usual.

Minute amounts of dissolved copper also catalyze atmospheric oxidation (13), and since the ordinary distilled water, reasonably clean laboratory apparatus, and many c.p. reagents contain significant quantities of copper, the hazard from this factor is relatively great. It is reasonably certain that reports of finding appreciable amounts of the vitamin in the reversibly oxidized state in natural fresh tissues have been in error because of secondary oxidation. The apparent rôle of glutathione in maintaining the vitamin in a reduced state *in vitro* and *in vivo*, and the secondary irreversible change (*in vitro*) of dehydroascorbic acid in neutral solution are items of particular importance in relation to this view (13-15).

The cellular structures in some tissues are disrupted more rapidly by trichloroacetic acid (4 to 8 per cent) than by metaphosphoric or acetic acids, and the former also exerts a better deproteinizing action. However, in the presence of trichloroacetic acid alone the vitamin is oxidized at an appreciable rate and may thus introduce a serious error, particularly in delayed titrations. Occasional lots of the acid are very bad in this respect, and should be discarded. The observed oxidative effect was not related to the hypochlorite or copper content of the acid.¹ The metaphosphoric acid (1 to 5 per cent) exerts a protective effect against both atmospheric and trichloroacetic acid oxidation.

In preliminary work it was found that the addition of 2 per cent

¹ The oxidation is accompanied by the liberation of Cl^- , analogous to the observed oxidation of $\text{Cu}^+ \rightarrow \text{Cu}^{++}$, reported by Neuberg and Kobel (16).

TABLE I

Stabilizing Effect of Metaphosphoric Acid upon Vitamin C in Solution

Each solution containing 0.4 mg. of vitamin C per 5 cc. was exposed to the air at room temperature in a 50 cc. Erlenmeyer flask.

Solution	Per cent destroyed after			
	0.5 hr.	1.0 hr.	1.5 hrs.	2.0 hrs.
Acetic acid, 8%.....	0	0	0	0
“ “ 8%, + 0.01 mg. Cu ⁺⁺	8	18	28	37
“ “ 6%, + 0.01 “ “.....	0	0	0	0
+ HPO ₃ , 2%.....	0	0	0	0
HPO ₃ , 2%.....	0	0	0	0
“ 2%, + 0.01 mg. Cu ⁺⁺	0	0	0	0
CCl ₃ COOH, 8%				
Sample A.....	0	0		9
“ B.....	46	74	90	100
“ C.....	95			
CCl ₃ COOH (B), 8%, + HPO ₃ , 2%.....	0	0	0	0
“ “ 8%, + “ 2%, + 0.01 mg. Cu ⁺⁺	0	0	0	0
Water, distilled supply.....	75	86		
“ tap.....	23	57	95	
“ redistilled from quartz.....	0	0	0	0

TABLE II

Influence of Extractants on Vitamin C Titration Values (Mg. per Gm.)

Animal and plant tissues	Extractant				
	HPO ₃ , 2 per cent	CCl ₃ COOH, 8 per cent	CH ₃ COOH, 8 per cent	8 per cent CH ₃ COOH + 2 per cent HPO ₃	8 per cent CCl ₃ COOH + 2 per cent HPO ₃
Beef adrenals.....	1.07	0.92	1.00	1.11	1.12
“ spleen.....	0.44	0.41		0.46	0.48
“ pancreas.....	0.11	0.10	0.12	0.13	0.14
Green peppers.....	1.72	1.33	1.78	2.08	2.01
Potatoes.....	0.10	0.07	0.08	0.10	0.10
Turnips.....	0.36	0.26	0.33	0.38	0.38

metaphosphoric acid to standard solutions of vitamin C in specially redistilled water, ordinary distilled water, tap water, acetic acid solution, and trichloroacetic acid solution, delayed oxidation when the flasks were exposed to air at room temperature. This was also true when small amounts of copper salts were added, as indicated in Table I.

The results given in Table II indicate clearly the advantage of using 2 per cent metaphosphoric acid with 8 per cent trichloroacetic acid or acetic acid when grinding tissue for extraction and titration of the vitamin. Each value given in Tables I and II represents an average of six or more determinations which were in close agreement.

SUMMARY

The titration procedure of Bessey and King for the determination of vitamin C has been modified to include the presence of 2 per cent metaphosphoric acid with acetic acid or trichloroacetic acid during extraction and titration. The modified procedure is advantageous for work with both plant and animal tissues.

Metaphosphoric acid in approximately 2 per cent concentration, as suggested by Fujita and Iwatake, serves to protect vitamin C in solution against atmospheric oxidation, even in the presence of added copper, and also exerts protective action against oxidation in the presence of trichloroacetic acid. The rate of reaction with 2,6-dichlorophenol indophenol is not appreciably affected by the presence of metaphosphoric acid.

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THE CHEMICAL ESTIMATION OF THEELIN WITH DIAZOBENZENESULFONIC ACID

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Since the elucidation of the chemical structure of the estrogenic hormones, several attempts have been made to replace the biological assay of these hormones by a simple chemical method of estimation. The first quantitative chemical method for the estimation of theelin was that proposed by Kober (1). This method is essentially an application of the Liebermann-Burchard sterol reaction to the quantitative assay of the hormone and is based on the reaction of estrin¹ with concentrated sulfuric acid containing phenol to give a red color. This color is compared with a standard of cresol red. The method of Kober is designed for the estimation of pure crystalline theelin; it is not adapted to the estimation of estrin in biological fluids.

Recently the Kober test has been investigated and modified by Cohen and Marrian (2), who developed it into a quantitative method for the estimation of theelin and theelol in human pregnancy urine. The red color given by the hormones is measured in a Lovibond tintometer and estimation is made by reference to a curve made with the pure hormone. These authors have shown that the values obtained by this method agree well with those obtained by bioassay.

1 year later, Cartland *et al.* (3) published a modification of the Kober test much simpler than that of Cohen and Marrian but suitable only for the estimation of relatively pure estrin extracts. The color comparison against a pure theelin standard is made

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¹ The term estrin is used in this paper to include both theelin (keto-hydroxyestrin, estrone) and theelol (trihydroxyestrin, estriol).

either grossly or in the colorimeter. Like Cohen and Marrian these workers found the results to be in good agreement with the bioassay.

Zimmerman (4) has recently discovered a color reaction of the sex hormones with *m*-dinitrobenzene. This reaction is specific for the ketone group and, hence, is given by progesterone, androsterone, and theelin but not by theelol. The reaction is quantitative; potassium dichromate or potassium permanganate solutions or mixtures of these two serve as color standards. The test readily detects 20 micrograms of theelin, and, like the Kober test, has been applied only to solutions of the pure hormones.

The present paper reports an investigation showing that theelin can be estimated much more easily than with any of these methods by coupling it with diazobenzenesulfonic acid to give an azo dye. The technique is a modification of that utilized by Hanke and Koessler (5) for the microcolorimetric estimation of phenols. Since the reaction depends on the phenolic hydroxyl of the estrin, it is, unlike the dinitrobenzene reaction, given by both theelin and theelol.

The diazo reaction of theelol was first noted by Harington and Schüpbach (6) who used diazotized *p*-nitroaniline as color reagent. We have used this reagent as well as diazotized sulfanilic acid with aqueous solutions of theelin; with small quantities of the hormone (5 to 50 rat units) the *p*-nitroaniline gives yellow colors which are difficult to match and do not appear to follow Beer's law as well as does the red azo dye given with diazotized sulfanilic acid.

Our procedure is very similar to Hanke and Koessler's but because of an important modification we think it is well to describe it briefly in its entirety.

Reagents—

Stock sulfanilic acid. Dissolve 4.5 gm. of sulfanilic acid (Eastman, No. 238) in 500 cc. of water containing 45 cc. of concentrated HCl.

Stock sodium nitrite, 5 per cent. Dissolve 25 gm. of Baker's Analyzed 86.3 per cent sodium nitrite in water and dilute to 500 cc.

Sodium carbonate, 1.1 per cent. Dissolve 5.5 gm. of Baker's Technical anhydrous sodium carbonate in water and dilute to 500 cc. Preserve in Pyrex glass vessels.

Sodium hydroxide, 10 per cent. Dissolve 50 gm. of Baker's

Analyzed sodium hydroxide (pellets) in water and dilute to 500 cc.

Stock β -naphthol, 0.01 per cent. The pure sublimed β -naphthol of Mallinckrodt was resublimed (m.p. 122.5°) and 0.1 gm. of this dissolved in water and diluted to 1000 cc.

Dilute β -naphthol (the standard). Dilute 10 cc. of the stock β -naphthol to 100 cc. with water.

Preparation of p-Diazobenzenesulfonic Acid—The sulfanilic acid is diazotized by mixing 1.5 cc. of the stock solution with 1.5 cc. of the 5 per cent sodium nitrite in a 50 cc. volumetric flask. The mixture is then immersed in an ice bath and after 5 minutes 6 cc. more of the nitrite are added. Again immerse in the ice bath for 5 minutes and then dilute with water to the mark. This solution is kept in the ice bath and is not used for at least 15 minutes after dilution with water nor after 24 hours.

Theelin Solution—The theelin solution (Parke, Davis, Rx-853,524) used in these experiments was "an aqueous solution of pure crystalline theelin . . . containing 50 rat units per cc., the assay having been checked not only in our laboratory but also by Dr. Doisy at St. Louis University".² This solution which was alkaline "to the extent of 0.005 N"² was neutralized by us before use by the addition of 0.1 N HCl.

Procedure

To $(1-x)$ cc. of water add 5 cc. of 1.1 per cent sodium carbonate, 2 cc. of diazobenzenesulfonic acid, and mix. Exactly 30 seconds after the diazotized sulfanilic acid has been added, add x cc. of the theelin solution and then 0.5 cc. of 10 per cent NaOH. 1 cc. (0.01 mg.) of the dilute β -naphthol standard is simultaneously treated, exactly as the theelin. The color comparison is made in the Duboscq colorimeter at the end of 3 to 5 minutes.

This procedure differs from that of Hanke and Koessler in the final addition of the NaOH and in the use of β -naphthol instead of a dye solution as a color standard. We have found that the final addition of NaOH accomplishes two things. First, the β -naphthol coupling is light brown before the NaOH is added and cannot be matched against the predominantly red color given by theelin, but

² Personal communication from Dr. Oliver Kamm (Parke, Davis and Company) who kindly furnished this material.

the addition of the NaOH changes this brown to a color identical with that given by theelin. Second, the NaOH stabilizes the dye produced so that its color is permanent for at least 1 hour, whereas without it the color fades to a very pale yellow-green in about 15 minutes.

Results

Quantities of theelin solution representing from 5 to 50 rat units were coupled by this procedure and matched with the color given by the β -naphthol standard. The results, which are summarized in Table I, show that the amount of color produced is proportional to the quantity of theelin.

TABLE I
Colorimetric Values of Theelin Compared with β -Naphthol Standard

Theelin	Average of 5 consecutive mm. readings of standard with theelin placed at 20 mm.	Average of readings minus value of blank (1.84 mm.)
<i>rat unite</i>	<i>mm.</i>	<i>mm.</i>
0	1.84	
5	3.76	1.92
10	5.44	3.60
15	7.64	5.80
20	9.72	7.88
25	11.84	10.00
30	13.74	11.90
35	15.92	14.08
40	17.52	15.68
45	19.74	17.90
50	21.92	20.08

A blank run on the reagents in the absence of theelin (1 cc. of distilled water replacing the theelin solution) develops a pale yellow color which, as is shown in the first line of Table I, has an intensity equivalent to about 1.8 mm. of the standard β -naphthol solution. The figure obtained when the value of this blank is subtracted from the average of five consecutive readings is given in the last column.

The average of the differences in mm. between our highest and lowest readings is 1.4 mm. Our least variation in any series of consecutive readings is 0.7 mm., the greatest 2.8 mm., the variations, in general, following a linear increase.

DISCUSSION

It would be most interesting to determine how much β -naphthol is colorimetrically equivalent to a given quantity of theelin, for with this information one would then have a readily available and inexpensive standard with which to estimate unknown quantities of theelin. However, to do this requires a weighable amount of the pure crystalline hormone, something which, as yet, we have been unable to obtain. In its absence, it occurred to us that perhaps (since theelin structurally is essentially aromatic tetrahydro- β -naphthol with two additional saturated rings attached to the hydrated ring and since both compounds produce the same colored azo dye) the reaction might be molecularly quantitative; that is, that equal moles of theelin and of β -naphthol would give equivalent amounts of color. To test this hypothesis we decided to refer to the tables of Hanke and Koessler where the azo dyes of varying amounts of closely related phenols were matched against a common color standard. If this hypothesis were true, then equal moles of *p*-cresol, *p*-hydroxyphenylacetic acid, and *p*-hydroxyphenylpropionic acid would require the same number of mm. of standard Congo red solution to match them.

An examination of Hanke and Koessler's tables for these three compounds ((5) pp. 252-254) showed us that this was, within the limits of experimental error, indeed the case. For example, 10 micrograms of *p*-cresol (0.925×10^{-7} mole), 14 micrograms of *p*-hydroxyphenylacetic acid (0.921×10^{-7} mole), and 16 micrograms of *p*-hydroxyphenylpropionic acid (0.963×10^{-7} mole) all require 5 mm. of standard Congo red solution to match the red color they give when coupled with diazotized sulfanilic acid. The same thing can be verified with other quantities of these three phenols where identical mm. readings occur.

As far as we know, the stoichiometric nature of this reaction has not been pointed out before. It probably explains why Hanke and Koessler found that, "It is a curious coincidence that the tabular values are identical for both tyrosine and tyramine hydrochloride." Tyrosine has a molecular weight of 181 and their tyramine hydrochloride, which was 98 per cent pure, a molecular weight of 173.

Even with *p*-hydroxyphenyllactic acid, where some change in this behavior might be expected because of the introduction of an

asymmetric carbon atom into the side chain, we still find that 1 mole of the homologous phenols listed above is colorimetrically equivalent to 1.18 moles of this acid.

We therefore decided to calculate the quantity of theelin colorimetrically equivalent to β -naphthol on a molecularly equivalent basis, even though this were probably only an approximation, and to calculate from this the rat unit equivalence of theelin to see how this checked with the figure of Doisy *et al.* (7) of 1 mg. of theelin = 3000 rat units. We obtained from the following calculation, where 144 and 270 are the respective molecular weights of β -naphthol and theelin, $144:270 = 0.01:X$, where $X = 0.01875$ mg. of theelin = 0.01 mg. of β -naphthol. And since we have found that 0.01 mg. of β -naphthol is colorimetrically equivalent to 50 rat units (see Table I), a second calculation gives $0.01875:50 = 1.00:X$, where $X = 2666$ rat units = 1 mg. of theelin, which is in very good agreement with the figure of Doisy *et al.*

It can be seen from our results that as few as 5 rat units of theelin in a total of 8.5 cc. of solution can be estimated quantitatively. According to the foregoing calculation this would correspond to 1.8 micrograms, a sensitivity considerably greater than that of the dinitrobenzene reaction, the limit of which, according to Zimmerman, is 20 micrograms in a total of 0.18 cc. of solution.

Whereas our method is at present suitable only for the estimation of pure theelin solutions, we believe that it should be possible to devise a method of estimation in the presence of interfering substances occurring in biological fluids as phenols, aromatic hydroxy acids, aromatic amines, and imidazoles on the basis of a quantitative separation of these substances along the lines worked out by us (8) for the detection of estrin in pregnancy urine.

SUMMARY

A colorimetric procedure for the estimation of theelin is described. The method is based on the coupling of theelin with *p*-diazobenzenesulfonic acid to give a red azo dye which is compared with a standard solution of β -naphthol coupled in the same manner. Certain observations concerning the stoichiometric nature of this reaction are pointed out.

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THE REACTION OF FORMALDEHYDE WITH AMINO ACIDS

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The studies here recorded form part of an investigation of the reactions that occur when diphtheria toxin is converted into toxoid and may also prove of value in further studies of the action of toxin in the tissues. When formaldehyde is added to toxin, it reacts with the various amino compounds present in the mixture. While the reaction between amino acids and formaldehyde has been extensively studied, the experimental conditions employed have usually been different from those which prevail in the formation of toxoid from toxin. The usual procedure at present for the preparation of diphtheria toxoid is to treat the toxin with from 0.3 to 0.4 per cent formalin (equivalent to 0.12 to 0.16 per cent of formaldehyde) at 37–39° for 4 weeks or more, the initial reaction being in the range of pH 7.6 to 8.4. The optimum concentration of formaldehyde varies with the medium in which the toxin is produced and appears to be related to the amino nitrogen content of the crude toxin; it is less than the amount theoretically equivalent to the amino nitrogen present. It is obvious that the results of experiments at high temperature, high pH, or high concentrations of formaldehyde have only an indirect bearing on the toxin problem.

A few investigators have reported studies of the reaction under conditions more or less resembling those of toxoid formation. Holden and Freeman (1) incubated several amino acids with 0.5 per cent formaldehyde in 0.1 *N* sodium hydroxide at 37° for 36 days. They found no reaction in acid solution, while in 0.1 *N* alkali there was a rapid initial reaction, followed by a further slow decrease in amino nitrogen. They also found that the simple amino acids were markedly less reactive than certain proteose and peptone preparations previously studied by Freeman (2). Gubareff

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and Bystrenin (3) investigated the effect of varying initial alkalinity and concentration of formaldehyde on the speed and completeness of the reaction with glycine at 38°. Levy (4) studied the titration curves of a number of amino acids at 30° and concluded that the amino group may be associated with either 1 or 2 molecules of formaldehyde, but the complex containing 2 molecules apparently forms only in the presence of a large excess of formaldehyde. Tomiyama (5) determined equilibrium constants for the reaction of formaldehyde with glycine, alanine, and proline at 25° in a pH range from 8.0 to 10.0 and found that a constant was obtained only on the assumption that the combining ratio was 1. Both Levy and Tomiyama consider that their data indicate the formation of molecular compounds.

The purpose of the present study was to obtain data on the behavior of amino compounds of known constitution when treated with formaldehyde under conditions similar to those used for the production of diphtheria toxoid. The following substances were studied: (a) amino acids—glycine, alanine, lysine, cysteine, aspartic and glutamic acids, histidine, tryptophane, and arginine; (b) guanidine; (c) two synthetic dipeptides—alanylglycine and glycylalanine; and (d) peptone and a crude diphtheria toxin.

EXPERIMENTAL

The amino acids and guanidine were purchased from the Eastman Kodak Company. The peptone used was Difco proteose. The toxin was a crude diphtheria toxin produced in infusion-free medium prepared with the same peptone (6). Alanylglycine and glycylalanine were synthesized and purified according to the methods of Fischer (7).

Methods

The substances to be studied were dissolved in 0.05 M phosphate buffer and the hydrogen ion concentration was adjusted to the desired level by the addition of 1 N sodium hydroxide. The pH range employed was 7.8 to 8.4 (determined colorimetrically). In most cases the concentration of the test substance was 0.05 M. Formaldehyde was added in an amount approximately equivalent to the amino nitrogen present. The solution was then made up to volume, mixed, and analyzed as soon as possible. When the reaction was very rapid, as in the case of cysteine, the initial values were determined by the analysis of control solutions. Toluene

was added as a preservative, and the solutions were incubated in paraffin-sealed bottles at 39°. At intervals (usually after 2 and 24 hours, 7, 14, 21, and 28 days) samples were withdrawn and analyzed for (a) amino nitrogen by the Van Slyke microprocedure, (b) free formaldehyde, and (c) reversibly bound formaldehyde. Control solutions both of the amino compounds and of formaldehyde were found to be stable on incubation, except in the case of cysteine, which is gradually oxidized.

Free Formaldehyde—The method was essentially that recommended by Velluz (8) and depends on the fact that the precipitation of formaldehyde by dimethyldihydroresorcinol (methone) can be made quantitative by the choice of appropriate conditions. As our method differed in some details from that of Velluz, it will be described here. The quantities used were adapted to micro and semimicro weighing.

The sample to be analyzed, containing from 0.4 to 2 mg. of formaldehyde, was mixed with 20 cc. of a saturated aqueous solution of methone (4.5 gm. per liter). If necessary, 2 or 3 drops of 10 per cent acetic acid were also added to bring the final pH of the mixture in the range 4.4 to 5.0, since it was found that in the presence of amino compounds the pH must be 5.2 or lower to insure quantitative precipitation of free formaldehyde. After the mixture had stood at room temperature for 4 hours, the precipitate was collected on a microfilter, washed with water, dried for 2 hours at 110°, and weighed. 1 mg. of formaldimethone is equivalent to 0.1027 mg. of formaldehyde.

Warm acetone was used to wash the precipitate from the filter after weighing. When the solutions contained protein or peptone, it was found convenient to dissolve the wet precipitate from the filter with warm acetone, remove the acetone in a water bath, resuspend the purified precipitate in water, then filter, dry, and weigh. In agreement with Velluz, we found the method to be accurate to about 3 per cent.

Reversibly Bound Formaldehyde—In order to study the reversal of the reaction between formaldehyde and amino compounds, the formaldehyde determination was modified. The solution to be analyzed was mixed with methone and the mixture was kept at 39° for 3 days before it was filtered and the precipitate weighed. Usually the amount of formaldimethone thus found was greater

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than that found in the free formaldehyde determination, indicating that exposure to methone at 39° had caused an appreciable splitting of the formaldehyde-amino compounds which had formed. The 3 day period does not necessarily give the maximum reversal possible with this reagent, since in a few cases longer periods of standing at 39° gave slight further increases in the amount of formaldimethone precipitated. For comparative purposes, however, it was considered more important to adopt a constant standardized procedure than to attempt a determination of the maximum reversal.

It was found in some control experiments with solutions of glycine and peptone which had reacted with formaldehyde that acidification of the reaction mixture to pH 4.8 with subsequent incubation at 39° was insufficient to reverse the reaction in the absence of methone.

Results

The experimental data are given in Tables I and II. It will be seen that the compounds studied varied greatly in the speed and completeness of their reaction with formaldehyde. The differences were, in general, in the same order as those found by Holden and Freeman, but were somewhat more marked at the higher hydrogen ion concentration employed by us. On continued incubation of the amino compounds with formaldehyde there was a steady decrease in the percentage of the total combined formaldehyde which could be split off by methone. There were four exceptions: (a) the two dipeptides, with which no reversal of the reaction was detected at any test period; and (b) arginine and guanidine, with which there was no significant change in the percentage of reversibly bound formaldehyde during the whole time of incubation. Table III shows the variations in reversibly bound formaldehyde, calculated from the data of Table I. From the gradual change in stability towards methone, it appears most probable that in these cases the reaction proceeds in two stages. The initial reaction may be quite rapid and is rather easily reversible. In the second stage, characterized by increased stability toward methone, the initial reaction product is probably transformed into a more stable compound by a secondary reaction or possibly a rearrangement.

These two stages are further illustrated by the special cases of histidine and tryptophane. Figs. 1 and 2 represent graphically the data for histidine and tryptophane which are given in Table I; the curves for reversibly bound formaldehyde are omitted. The apparent combining ratio (moles of HCHO to moles of NH_2) varies strikingly and reaches the value of 1 only as the reaction approaches completion. It can scarcely be supposed that the true combining ratio varies in such a way, but it is quite reasonable to assume that the initial reaction product is more stable toward methone than toward nitrous acid (tryptophane) or *vice versa* (histidine), and that this initial product is gradually transformed into a second compound which is stable toward both reagents. In this case the apparent combining ratio would vary in the manner found by experiment.

The reaction product of tryptophane and formaldehyde crystallized from the solution and was identified as 3, 4, 5, 6-tetrahydro-4-carboline-5-carbonic acid. This substance was synthesized by Jacobs and Craig by the condensation of tryptophane and formaldehyde in acid solution (10). When our product was compared with a sample of the carboline acid obtained from Dr. Jacobs, the two substances melted simultaneously with decomposition at 306° (uncorrected) and there was no depression of the melting point when the two were mixed. The yield of the carboline compound under the conditions of our experiment was nearly quantitative. The fact that this condensation takes place so readily under physiological conditions is of considerable interest, especially in view of the results of Hahn and his coworkers (11) who have prepared numerous 4-carboline derivatives from tryptamine at physiological temperatures in the pH range of 3.4 to 6.2. We are much indebted to Dr. Jacobs for the opportunity to compare the two compounds.

Holden and Freeman state that formolized histidine also crystallized, but under the conditions of our experiments the product of the reaction between histidine and formaldehyde did not separate from solution.

Equilibria in Alkaline Solutions—The experiments on the reversal of the reaction by methone were all carried out in acid solution (pH 4.4 to 5.0), since this acidity was necessary for the condensation of methone with formaldehyde. In slightly alkaline

Aspartic acid†	Amino N	61.5		61.5	61.9	61.8	61.0	60.2	7.8	7.6
Glutamic " ‡	" "	48.7		48.5	48.4	47.8	46.6	45.4	8.0	7.8
Peptone (2%)	" "	50.4	38.6	29.2	22.2	22.2	20.9	20.0	7.8	7.4
	Free HCHO	132.0	105.0	71.6	51.5	45.9	41.0	37.8		
	HCHO 39°		118.0	88.0	66.4	62.9	57.7	50.1		
Toxin	Amino N	68.5	53.7	46.5	37.2	39.5	40.4	38.0	8.4	8.2
	Free HCHO	115.0	84.0	54.2	27.5	33.8	28.6	24.5		
	HCHO 39°		117.0	93.0	77.0	65.7	58.0	47.8		
		10 min.	1 hr.	2 hrs.	4 hrs.	5 hrs.	8 hrs.	13 hrs.	24 hrs.	4 days
Histidine	Amino N	56.0	13.0	8.2	2.4		0.0		8.2	7.6
	Free HCHO	132.0	119.0	94.5	33.5		11.8			
	HCHO 39°			95.5	32.5		12.1			
Tryptophane	Amino N	42.5	37.9	30.0		20.0	8.8	6.5	1.7	7.6
	Free HCHO	85.0	41.8	32.0		15.9	6.9	6.0	Trace	
	HCHO 39°		49.0	37.4	29.3	18.0	8.0	5.5		

The line "HCHO 39°" gives the total amount of HCHO found after incubating the sample with methone for 3 days as described in the text.

* At this concentration of cysteine the reaction had reached equilibrium in less than 15 minutes. The irregular results obtained later may be partly due to the instability of the acid.

† Glycylalanine, like glycylglycine (9), gives abnormal results in the Van Slyke analysis; hence, the amino nitrogen figures are not reported. In this experiment the pH at first fell rapidly and was readjusted to 8.2 after 4 hours.

‡ These two experiments were carried out at 36-37° instead of 39°.

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TABLE II
Percentage of Components Reacting

		2 hrs.	24 hrs.	4 days	7 days	11 days	14 days	17 days	21 days	28 days	Initial concentration
											<i>mole per l.</i>
Histidine	Amino N	90	100								0.040
	HCHO	52	91								0.044
Tryptophane	Amino N	29	85	96							0.03
	HCHO	70	93	100							0.029
Cysteine*	Amino N	71	70							61	0.025
	HCHO	71	71		63		55		58	63	0.028
Arginine	Amino N	23	32	40		43					0.05
	HCHO	48	65	76		77					0.05
Guanidine (0.045 M)	"		14		18		16		17	17	0.042
Glycine	Amino N	6.4		10		15		16		22	0.05
	HCHO	5.6		10		17		22		25	0.048
Alanine	Amino N			6				15		20	0.05
	HCHO			7.6				23		27	0.048
Alanylglycine	Amino N	0	23		54		67		69	73	0.05
	HCHO	0	21		56		67			82	0.05
Glycylalanine (0.05 M)†	"			14				37		60	0.05
Lysine (0.05 M)	Amino N	0	11		23		30		31	32	0.10
	HCHO	15	30		58		73		78	82	0.055
Aspartic acid‡	Amino N		0		0		0		1	<3	0.043
Glutamic " ‡	" "		0		0		<2		4	7	0.036
Peptone (2%)	" "	23	42	49	56		56		59	60	0.037
	HCHO	21	46	58	61		65		69	71	0.044
Toxin	Amino N	22	32	42	46		42		41	44	0.049
	HCHO	27	53	68	76		71		75	79	0.038

* At this concentration of cysteine the reaction had reached equilibrium in less than 15 minutes. The irregular results obtained later may be partly due to the instability of the acid.

† Glycylalanine, like glycylglycine (9), gives abnormal results in the Van Slyke analysis; hence, the amino nitrogen figures are not reported. In this experiment the pH at first fell rapidly and was readjusted to 8.2 after 4 hours.

‡ These two experiments were carried out at 36–37° instead of 39°.

solution it was found that methone not only did not cause any reversal but did not prevent the continued condensation of formaldehyde with amino groups. It could not be assumed that the stability relationships revealed by the methone reaction necessarily corresponded to those existing in alkaline solutions because of the marked effect of changes in hydrogen ion concentration on the reaction between amino groups and formaldehyde. If the rela-

TABLE III
Percentage of Reversibly Bound Formaldehyde

	2 hrs.	24 hrs.	4 days	7 days	11 days	14 days	17 days	21 days	28 days
Cysteine.....		81		77		71		64	53
Glycine.....	100		85*		75		53		39
Alanine.....			63				37		13
Lysine.....	100	47		28		20		13	9
Peptone.....	48	27	26	19		20		18	13
Toxin.....	100	64	51			39		34	26
Arginine†.....	100	96	100		100		97		97
Guanidine.....		50		52				47	
Alanylglycine.....		0		0		0			0
Glucylalanine..			0				0		

The reversibly bound formaldehyde is the difference between the amount found on treating with methone for 3 days at 39° and that found in 4 hours at room temperature; this is recorded as a percentage of the total amount of formaldehyde which had reacted (initial value minus free formaldehyde value).

This table represents the same series of experiments as Tables I and II.

* Average of three experiments.

† Average of two experiments.

tionships in alkaline solution can be predicted from the results of the methone reaction, it is evident that, in a mixture containing insufficient formaldehyde to react with all the amino groups, alanylglycine, for example, will gradually remove formaldehyde from its compounds with such acids as cysteine and arginine, and this will result in a decrease of reversibly bound formaldehyde in the mixture. This may serve as an example of the complex equilibria in a mixture of formaldehyde with crude toxin.

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0.1 M solutions of cysteine and of arginine were prepared in phosphate buffer at pH 8.2 and allowed to react with equivalent quantities of formaldehyde for 24 hours at 39°. The solutions were then readjusted to pH 8.2. One portion of each solution was

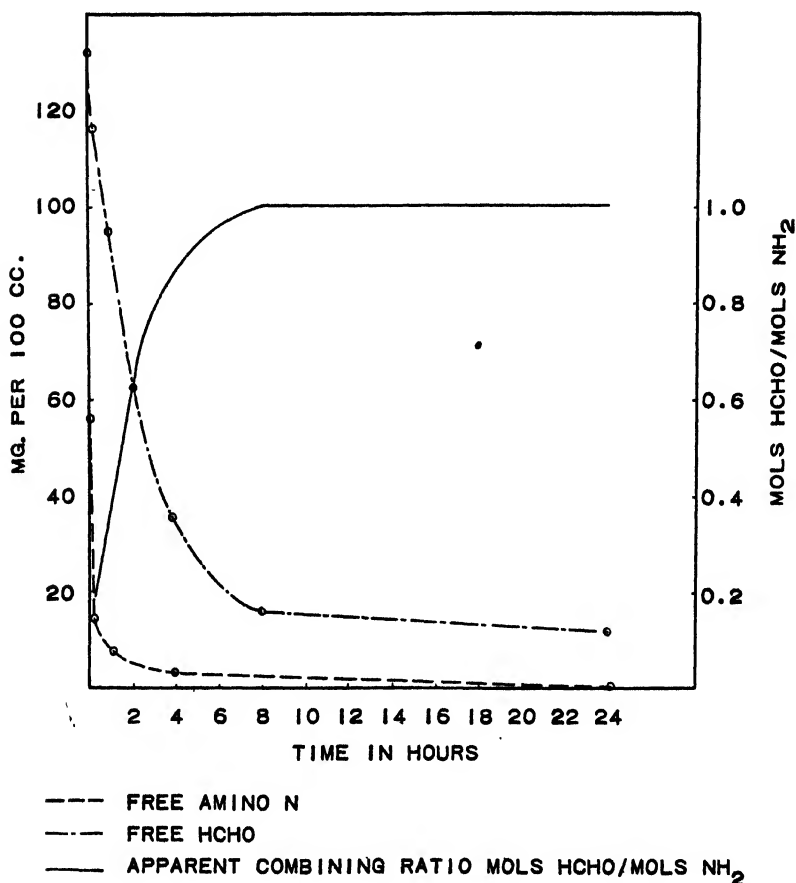


FIG. 1. Reaction of histidine with formaldehyde

mixed with an equal volume of 0.1 M alanylglycine in phosphate buffer, and the remainder was diluted to 0.05 M with phosphate buffer and incubated as a control. All solutions were incubated at 39° and analyzed at intervals as previously described. The

results are summarized in Table IV. It will be seen that there was a marked decrease in reversibly bound formaldehyde in the mixtures containing alanylglycine as compared with the controls containing cysteine or arginine alone. We may safely assume,

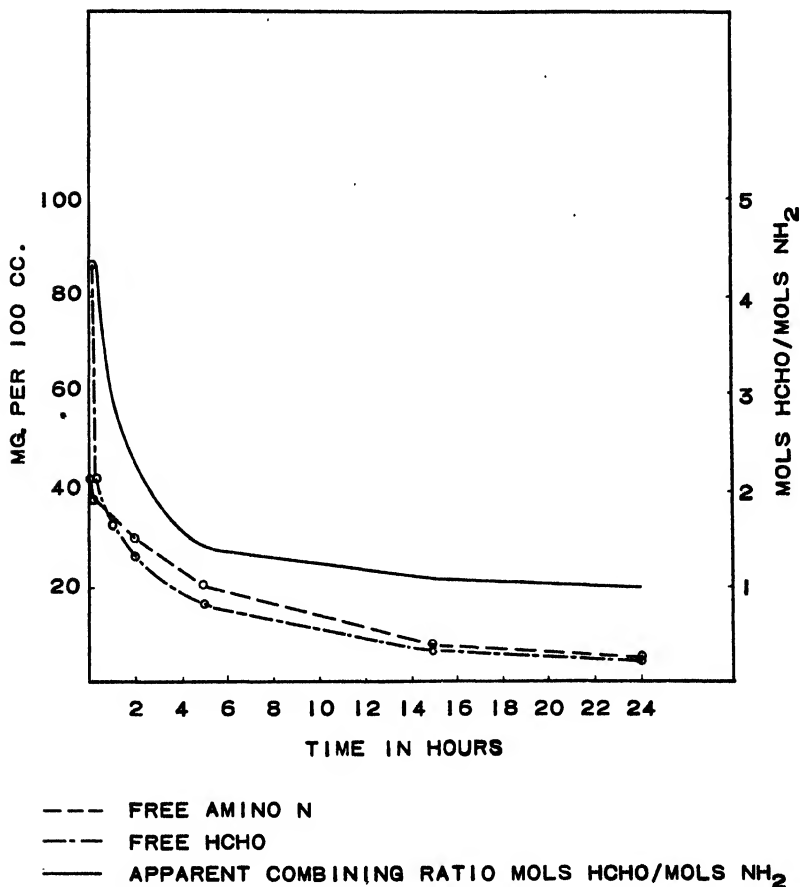


Fig. 2. Reaction of tryptophane with formaldehyde

then, that the behavior of the different formaldehyde-amino compounds toward methone shows their relative stability in alkaline solutions. It is interesting to compare this series of experiments with one reported by Holden and Freeman (1), who

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were able to bring about a partial reversal of the combination of formaldehyde with a metaprotein by incubating with histidine or lysine.

DISCUSSION

It is evident that the analytical methods used in the present study are not capable of giving a complete picture of the reactions taking place. In considering the experiments on histidine and tryptophane, we were forced to conclude that the first products formed were unstable under the conditions of analysis. Obviously

TABLE IV
Equilibria in Alkaline Solutions

	Initial pH*	Final pH	Reversibly bound HCHO (mg. per 100 cc.)					
			Initial value*	Time after mixing				
				24 hrs.	1 wk.	2 wks.	3 wks.	4 wks.
Alanylglycine 0.05 M + arginine 0.05 M.	8.2	7.4	86.3	76	38.9	25.6	20.4	17.6
Arginine 0.05 M.	8.2	7.6	83	92.6	96	90	92.4	94
Alanylglycine 0.05 M + cysteine 0.05 M.	8.2	8.2	101	82	64.9	41.9	23.8	13.4
Cysteine 0.05 M.	8.2	8.2	97	89	73	65.5	60.5	56.5

* In this case the initial value is that found immediately after mixing the solutions of the two amino acids, after the cysteine and arginine solutions had reacted with formaldehyde for 24 hours.

it is possible that the same may be true at least in the initial stages of the other experiments. This does not invalidate the use of the data for comparative purposes so long as it is emphasized that the terms "free amino nitrogen" and "free formaldehyde" are used in a relative rather than an absolute sense and are defined by standardized conditions of analysis.

The acids which show the greatest affinity for formaldehyde—histidine, tryptophane, arginine, cysteine (and, in the experiments of Holden and Freeman, tyrosine)—are those which have other functional groups in the molecule in addition to the amino and carboxyl groups. The second carboxyl group in aspartic

and glutamic acids decreases the reactivity. The simple amino acids, alanine and glycine, are markedly less reactive than the two dipeptides prepared from them. In the case of arginine, the analytical data indicate 2 moles of formaldehyde reacting in 0.05 M solution for each mole of arginine. Since guanidine itself combined with formaldehyde under similar conditions, it seems probable that the guanidine group of arginine also reacts with formaldehyde, although Sørensen (12) found that this group could not be determined by the formol titration method.

With regard to the mechanism of the reaction, it may be recalled that Hahn and his coworkers (11) found evidence of two stages in the reaction between tryptamine and substituted pyruvic acids. They considered the first step to be the formation of an addition compound which then loses water to form the carboline ring. It seems highly probable that the mechanism of the reaction between tryptophane and formaldehyde is similar and that the unstable compound indicated by our data is the hydroxymethyl compound which splits off water, yielding the stable carboline acid. It is interesting also to compare our results with those of Tomiyama (5). A calculation from Tomiyama's equilibrium constant for the reaction between glycine and formaldehyde indicates that at the concentrations used by us nearly 50 per cent of the formaldehyde would be associated with glycine as a molecular compound at 25°, while by chemical methods we find only about 10 per cent combined even after 4 days at 39°. Evidently the two sets of data do not represent the same reaction; the molecular compound detected by physicochemical methods must be immediately dissociated when chemical methods of analysis are used.

We suggest that, under the experimental conditions described by us and, therefore, also during the production of toxoid, there may be at least three stages in the combination of formaldehyde with amino groups: (a) a loosely associated molecular compound; (b) a labile chemical compound, possibly a hydroxymethyl or methylene derivative, indicated by the reversible stage in our experiments; and (c) a stable compound probably formed by further reaction or rearrangement of compound (b). In studies of toxoid production it is evidently insufficient to draw a distinction between "free" and "bound" formaldehyde, as the "bound" formaldehyde may be either reversibly or irreversibly combined,

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while some of the "free" formaldehyde may be loosely associated with amino groups to form molecular compounds.

SUMMARY

1. Data are presented on the course of the reaction of formaldehyde with several amino acids and related compounds under conditions comparable to those in which diphtheria toxin is transformed into toxoid.

2. In most cases two stages in the reaction can be demonstrated: (a) a rapid, reversible reaction; (b) a slower, irreversible one. The reversal of the reaction has been studied in both acid and alkaline solutions.

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THE INTERACTION OF IODOACETIC ACID AND TERTIARY AMINES

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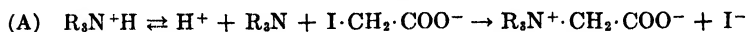
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Iodoacetic acid has been used extensively in the past few years in the study of a number of enzymatic reactions (1). It is often assumed more or less tacitly that if iodoacetic acid produces an effect in such a system this effect is due to its interaction with thiol groups. The reason for this is that the thiol group is the only one at present known which reacts rapidly enough with iodoacetic acid, under the special conditions imposed, to account for the effects observed when certain enzyme-substrate systems are treated with iodoacetic acid: the reaction must occur in dilute aqueous solution at physiological pH and temperature. Because of this situation any groups other than thiols which can react rapidly with iodoacetic acid under the conditions specified immediately become of biochemical interest.

In some previous work (2) on the interaction of iodoacetic acid and primary amines it was remarked that, although glycine reacts to give the compound $N(CH_2 \cdot COOH)_3$, there seems to be no tendency for an additional molecule of iodoacetic acid to react with this tertiary amine to form a quarternary ammonium salt. The same is true of a number of similar compounds of the general formula, $R \cdot N(CH_2 \cdot COOH)_2$. In view of the ease with which alkyl halides usually react with aliphatic tertiary amines to form quarternary ammonium salts this seemed peculiar and suggested that the condition which inhibited ammonium salt formation might be the accumulation of carboxyl groups in the neighborhood of the tertiary nitrogen atom. A study of the variation of the rate of this reaction with substitution on the reacting nitrogen has therefore been made. The group of compounds in Table I has been selected as an example. In this group the hydrogen of ammonia is progres-

sively substituted by methyl or carboxymethyl groups. Since the reaction was studied at pH 7.0, the amines are all in the form of ammonium ions. However, the reaction probably occurs with the deionized amine, as indicated by the work of Brdička (3). Thus the reaction scheme may be as follows:



where R may represent H, CH₃, or CH₂·COOH. Since the compounds in Table I vary in the total number of molecules of iodo-

TABLE I

Initial Rate of Reaction of Iodoacetic Acid or of Iodoacetamide with Various Amines at pH 6.9

The figures represent the number of mM of iodide ion formed per minute in 10 cc. of the reaction solution which contains each reactant at 0.115 M and 0.5 M of phosphate buffer, pH 6.9, temperature 30°.

	I CH ₂ COOH	I CH ₂ CO·NH ₂
NH ₃	0.000018	0.000029
NH ₂ ·(CH ₃)	0.000097	0.000084
NH·(CH ₃) ₂	0.00030	0.00023
N·(CH ₃) ₃	0.0013	0.00087
NH ₂ ·(CH ₂ ·COOH)	0.000075	0.000093
NH(CH ₃)(CH ₂ ·COOH)	0.00020	0.00020
N(CH ₃) ₂ (CH ₂ ·COOH)	0.00024	0.00021
N(CH ₃)(CH ₂ ·COOH) ₂	0.000038	0.000060
N(CH ₂ ·COOH) ₃	0.000019	0.000032
Blank, no amine	0.000013	0.000026
Creatine	0.000019	
Creatinine	0.00013	
Proline	0.00015	
Arginine	0.00016	
Histidine	0.00043	

acetic acid with which they can react and since it is not pertinent to the present study to examine the kinetics of the reaction in detail, only the initial velocity of the reaction has been determined. This velocity is recorded both for iodoacetic acid and iodoacetamide.

From Table I the following observations can be made: replacement of hydrogen or of carboxymethyl by a methyl group brings

about an increase in the speed of the reaction; substitution of hydrogen by carboxymethyl in most cases produces relatively less change in reactivity (for example, the speeds of reaction with NH_3 and $\text{N}(\text{CH}_2\cdot\text{COOH})_3$ are the same). Thus there is no reason to believe that accumulation of carboxyl groups about the reacting nitrogen has any specific effect. Finally, from Table I it appears that iodoacetamide behaves the same as iodoacetic acid. In this respect the amines differ from thiols, since with the latter the reactions are faster with iodoacetamide (4, 5).

Amines, then, are capable of reacting with iodoacetic acid and

TABLE II

Bimolecular Constants for Reaction between Iodoacetic Acid or Iodoacetamide and Compounds Listed in First Column

Each reactant is 0.115 M, pH 6.9, temperature 30°. To compare with the figures in Table I multiply by 0.13 (see "Experimental").

	I-CH ₂ ·COOH	I-CH ₂ ·CO·NH ₂
* Pyridine.....	0.038	0.017
Nicotinic acid.....	0.018	0.0090
" " amide.....	0.0096	0.0032
Nicotine.....	0.023	0.0098
Hexamethylenetetramine.....	0.54	0.18
Trimethyltrimethylenetriamine.....	0.074	0.028
Glutathione*.....	15	
Thioglycolic acid†.....	0.019	0.040

* The value given by Dickens (7) for pH 7.4 at 37°.

† The values given by Hellström (8) for neutral solution at 25°.

the rate of reaction is enormously influenced by substitution. However, even the fastest reacting amine of Table I, trimethylamine, reacts only about one-thousandth as fast as cysteine. In searching for other amines which might react more rapidly with iodoacetic acid, tertiary amines with no carboxymethyl groups attached to the reacting nitrogen seemed to be most promising. Pyridine is such an amine and actually does react with iodoacetic acid about 3 times as fast as trimethylamine. Pyridine is of particular interest in this connection, since a derivative, which on hydrolysis yields nicotinic acid amide, has recently been found by

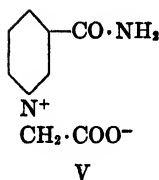
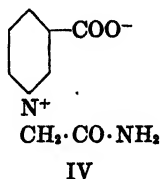
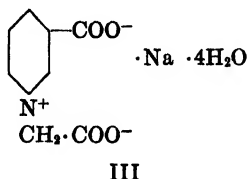
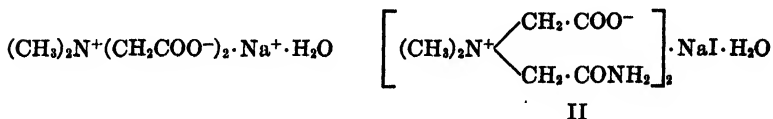
Warburg and coworkers (6) to be a component of the oxidation coenzyme. In Table II are given the bimolecular constants found for the reactions with pyridine and some of its derivatives.

Another group of amines studied is the products formed by the interaction of aldehydes with ammonia or amines. Only two examples need be mentioned, hexamethylenetetramine and trimethyltrimethylenetriamine. These two amines are the most rapidly reacting so far found and their velocity constants are included in Table II. For comparison some velocity constants for thiols are also included in Table II, taken from work of Dickens (7) and of Hellström (8). This shows that those amines of the pyridine group react with iodoacetic acid at speeds of the same order as that with which thioglycolic acid reacts, while in the case of hexamethylenetetramine a velocity constant half way between glutathione and thioglycolic acid is found. A further point brought out by Table II is the fact that this group of amines reacts more rapidly with iodoacetic acid than with iodoacetamide, thus differing from all thiols so far examined. This property becomes of special interest in view of the findings of Smythe (4) that fermentation is more inhibited by iodoacetic acid than by iodoacetamide.

In Table I there are also included a few amino acids which react quite slowly with iodoacetic acid.

In the work so far described amine and iodo compound have been mixed in aqueous solution and the rate of formation of iodide ion has been determined. The assumption is that a reaction of the form (A) occurs. The possible objection exists that these amines merely catalyze a hydrolysis of iodoacetic acid. The isolation of a number of the reaction products disposes of this objection. All of these products are betaines and have been obtained in crystalline form. Dimethylamine and chloroacetic acid give the compound (I). Dimethylglycine and iodoacetamide give a product which crystallizes from alcohol with sodium iodide (II). From nicotinic acid the two products (III) and (IV) are easily obtained. Isomeric with the latter is the product resulting from nicotinic acid amide and iodoacetic acid (V). Of special interest is a compound formed from hexamethylenetetramine and iodoacetic acid (VI). Because of the 4 nitrogen atoms in this amine it might be expected that more than 1 mole of iodoacetic acid could react with each

mole of tetramine, but, even when twice as much iodoacetic acid is used, the same product is obtained. In both of the double salts (II) and (VI) the iodine is completely ionic.



EXPERIMENTAL

To follow the rate of formation of iodide ion from the interaction of amines and iodoacetate or iodoacetamide Volhard's method is used. 10 mm of the amine together with enough acid or base to neutralize it are made up to 62 cc. with 0.5 M phosphate mixture (0.1 M KH_2PO_4 and 0.4 M Na_2HPO_4). To this are added 25 cc. of a solution containing 10 mm of sodium iodoacetate or iodoacetamide made up in the same phosphate mixture. Samples of 10 cc. are withdrawn at suitable time intervals and dropped into excess 0.05 M AgNO_3 solution containing 10 cc. of 8 M HNO_3 and the mixtures titrated with 0.05 M NH_4SCN with ferric alum solution as indicator. The reactions are run in a constant temperature room kept at $30^\circ \pm 0.1^\circ$. All pH values were determined with a glass electrode and referred to standard acetate solution taken as pH 4.6. The pH of all solutions was 6.9 ± 0.1 . For the determination of initial velocities the reactions were allowed to run about 10 per cent of their full course, six samples being taken in this interval. The bimolecular constants were determined by

allowing the reaction to run about 50 per cent of its full course, six or seven samples being taken, and k calculated from the relation: $k = (1/at) (x/(10a - x))$, where t is the time in minutes, x the mm of iodide ion formed in this time in 10 cc. of the reaction mixture, and a the initial molar concentration of the reactants, that is, 10 mm in 87 cc. or 0.115 M. To compare the bimolecular constants of Table II with the initial velocity values of Table I the relation, initial velocity (mm per cc. per minute) = ka^2 , is used. Since the initial velocity values are calculated for 10 cc. of solution, this becomes, initial velocity (mm per 10 cc. per minute) = $10ka^2 = 0.13k$. The velocity values of Table I are uncorrected for the hydrolysis of the iodo compound. To correct for this the blank, also given in Table I, must be subtracted.

Preparations

Of the compounds listed in Table I only three require special preparation. Dimethylglycine was prepared as described elsewhere (9). Methyl dicarboxymethylamine is prepared by a similar method, as follows: Half of a solution of 70 gm. of NaOH in 150 cc. of water is added to a solution of 80 gm. of $\text{Cl} \cdot \text{CH}_2 \cdot \text{COOH}$ in 150 cc. of water, the mixture being kept cooled. This solution is poured into 50 gm. of a 33 per cent aqueous solution of methylamine. This mixture is cooled and the rest of the NaOH solution is then added. After standing overnight the solution is evaporated to dryness, and the residue is extracted with a boiling mixture of 1000 cc. of alcohol and 110 cc. of water, the undissolved NaCl being filtered from the hot solution. After the filtrate is cooled on ice, a liquid product separates, the alcohol is decanted, and the remaining liquid is washed by decantation with three 50 cc. portions of absolute alcohol. The liquid residue is dissolved in 300 cc. of hot methyl alcohol containing 75 cc. of water, the mixture is filtered, and 1000 cc. of absolute alcohol and 500 cc. of acetone are added. A white crystalline product separates which is filtered off after cooling on ice. The product may be recrystallized from methyl alcohol as above. After drying *in vacuo* 30 gm. of a light fluffy product are obtained. This is $\text{CH}_3\text{N}(\text{CH}_2 \cdot \text{COONa})_2$.

Calculated, N 7.33, Na 24.08; found, N 7.38, Na 23.80

The tricarboxymethylamine is made as previously described (2), except that chloroacetic acid is used instead of iodoacetic acid.

The betaine, $(\text{CH}_3)_2\text{N}^+(\text{CH}_2\cdot\text{COO}^-)_2\cdot\text{Na}^+\cdot\text{H}_2\text{O}$ has been prepared either from dimethylglycine and iodoacetic acid, or more simply as follows: A solution containing 40 gm. of $\text{Cl}\cdot\text{CH}_2\cdot\text{COOH}$, 250 cc. of water, 44 gm. of Na_2CO_3 , and about 40 gm. of a 33 per cent aqueous solution of dimethylamine is heated at 80° for 2 to 3 hours. It is evaporated to dryness and the residue extracted with 500 cc. of boiling alcohol to which 50 cc. of water have been added. The mixture is filtered while hot; the filtrate deposits needle-like crystals which are filtered off after cooling on ice. They may be recrystallized from a hot mixture of 400 cc. of alcohol and 40 cc. of water. After drying *in vacuo* 12 gm. of product remain.

Calculated, N 6.96, Na 11.44, H_2O 8.96; found, N 6.48, Na 11.44, H_2O 8.98

The curious double salt (II) is easily prepared by suspending 5 gm. of the sodium salt of dimethylglycine in 15 cc. of alcohol and adding a solution of 8 gm. of iodoacetamide in 50 cc. of alcohol. The suspension rapidly clears up and a new crystalline product soon separates. After standing overnight this is filtered off and recrystallized twice from 100 to 150 cc. of hot alcohol; the clear solution, being cooled on ice, deposits a felt-like mat of crystals.

Calculated for (II). N 11.47, Na 4.71, I 26.02
Found. " 11.27, " 4.93, " 25.81

To prepare the nicotinic acid betaine (III) 2.5 gm. of nicotinic acid are dissolved in 10 cc. of water by adding 5 gm. of NaHCO_3 ; then 2.8 gm. of $\text{Cl}\cdot\text{CH}_2\cdot\text{COOH}$ are added and the solution allowed to stand at room temperature for a day or two. The product is precipitated by addition of 100 cc. of alcohol and, after cooling on ice, is filtered off. It can be recrystallized by dissolving in a hot mixture of 75 cc. of alcohol and 25 cc. of water. On cooling on ice it crystallizes in long, dense needles. It must be dried for several days *in vacuo* to get the constant tetrahydrate.

Calculated for (III). N 5.09, Na 8.36, H_2O 26.18
Found. " 5.12, " 8.45, " 26.12

The betaine (IV) is prepared similarly, except that on recrystallization, since it is more soluble, it is dissolved in a hot mixture of 30 cc. of alcohol and 10 cc. of water, and after complete solution and filtration, if necessary, 40 cc. of alcohol are added. After

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cooling on ice the product separates as a felt-like mat of fine needles. After drying several days in a vacuum desiccator it comes to constant weight.

Calculated for (IV), N 15.55; found, N 15.73

The isomeric betaine (V) is prepared similarly from nicotinic acid amide and iodoacetic acid but is precipitated with acetone instead of with alcohol. It is recrystallized by dissolving in about 100 cc. of hot methyl alcohol containing 10 cc. of water (for an amount beginning with 10 mm of nicotinic acid amide), filtered, and precipitated by slow addition of 200 cc. of acetone. The yellow product is again crystallized from 150 cc. of hot methyl alcohol containing 5 cc. of water. On chilling, long yellowish needles separate.

Calculated for (V), N 15.55; found, N 15.40

To make the betaine (VI), 2 gm. of iodoacetic acid are dissolved in a mixture of 2 cc. of water and 4 cc. of alcohol and 0.9 gm. of NaHCO_3 is added to neutralize it. Then 1.4 gm. of hexamethylenetetramine are added and the mixture allowed to stand at room temperature for 4 hours. 40 cc. of alcohol are added and after chilling on ice the crystalline product is filtered off. It can be recrystallized by dissolving in a hot mixture of 20 cc. of alcohol and 4 cc. of water, filtering, and adding 25 cc. of absolute alcohol. The long needles are filtered off and dried *in vacuo*.

Calculated for (VI). N 13.96, Na 5.72, I 31.60

Found. " 13.90, " 5.87, " 31.94

The trimethyltrimethylenetriamine was prepared as described by Brochet and Cambier (10).

All of the analytical part of this work has been done by Mr. G. Bitterlich.

SUMMARY

The reaction of iodoacetic acid and of iodoacetamide with a number of amines has been studied. As is to be expected, the speed of this reaction varies greatly with the groups attached to the amine nitrogen. Among the amines studied there are some

whose speed of reaction with iodoacetic acid approaches that of the more rapidly reacting thiol compounds. Therefore in studying the effect of iodoacetic acid on enzyme reactions the possibility of reaction with amino groups, particularly tertiary amines, cannot be ignored.

It is also shown that with some types of amines, iodoacetic acid reacts more rapidly than iodoacetamide. These amines thus differ from thiols studied so far, all of which react more rapidly with iodoacetamide.

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THE ISOLATION AND CHARACTERIZATION OF A STARCH POLYSACCHARIDE FROM THE LEAF TISSUE OF THE APPLE TREE (*MALUS MALUS*)*

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In a previous investigation conducted in this laboratory (1) a starch polysaccharide, apparently identical with the water-soluble component of the common cereal and tuber starches, was isolated from the woody tissue of the apple tree (*Malus malus*). Thus it appears that starches of widely different origin may contain a common water-soluble polysaccharide.

Through an application of the method (2) used above a crude starch polysaccharide was obtained from the leaf tissue of the apple tree (*Malus malus*), which was separated into a water-soluble and a water-insoluble fraction. The water-soluble fraction was an amorphous white powder, $[\alpha]_D^{30} = +172^\circ$, which reacted with aqueous iodine-potassium iodide to give a blue color that disappeared on heating and reappeared on cooling. As this material contained minor quantities of erythrodextrin and pentosan, it was further purified by forming the iodine-iodide complex, which after precipitation with half saturated ammonium sulfate (3) was decomposed with silver nitrate. This procedure yielded an amorphous white polysaccharide which was soluble in water and formamide and possessed a specific rotation of $[\alpha]_D^{30} = +185^\circ$. An aqueous solution of the purified starch polysaccharide produced a pure blue color with the iodine-iodide reagent. Elementary

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analysis indicated a compound of type formula $(C_6H_{10}O_5)_x$, and as pentosans were shown to be absent, it was evident that the polysaccharide was a polyhexosan.

Evidence as to the structural similarity of the starch polysaccharide of the apple leaf to that of the apple wood was obtained from hydrolytic studies. Dilute acid hydrolysis of the leaf starch polysaccharide yielded a solution whose reducing power, calculated as glucose, was 98 per cent of that required for a theoretical yield. The equilibrium rotation of the hydrolysate was $[\alpha]_D^{30} = +62^\circ$. The absence of *d*-fructose, *d*-mannose, and *d*-galactose and the exclusive presence of *d*-glucose in the acid hydrolysate were established by the usual methods (4). The kinetics of the hydrolysis of the leaf starch polysaccharide in 5 N sulfuric acid were studied at 80° and 90°. The constants found were $K_{80} = 2.2 \times 10^{-2} \text{min.}^{-1}$ and $K_{90} = 7.2 \times 10^{-2} \text{min.}^{-1}$.

Enzymatic hydrolysis of the leaf starch polysaccharide with barley malt diastase yielded a digest from which maltose was isolated in the form of its phenylosazone. The kinetics of the enzymatic hydrolysis of the leaf starch polysaccharide and a soluble potato starch polysaccharide (*e.g.*, the β -amylose fraction) were studied under comparable conditions. The velocity constant for the leaf polysaccharide was $K_{37} = 1.2 \times 10^{-3} \text{min.}^{-1}$, and for the potato polysaccharide $K_{37} = 1.5 \times 10^{-3} \text{min.}^{-1}$. Further, it was demonstrated that the leaf starch polysaccharide and the potato starch polysaccharide were hydrolyzed at approximately identical rates, even though different diastatic enzymes were employed.

From the above it is apparent that the starch polysaccharide isolated from the leaf tissue of the apple tree is a polyglucosan, similar, if not identical, in structure to the β -amylose component of common cereal and tuber starches and to the starch polysaccharide previously isolated from apple wood (1).

Since the experimental portion of this study was completed, Campbell (5) and Spoehr and Milner (6) have reported the isolation of various wood and leaf "starches."¹ A critical comparison

¹ It is of interest to note that in 1926 Dr. R. G. L. Beazeley isolated the so called transitory starch present in potato leaves. This work was mentioned in a lecture by Professor A. R. Ling of the Biochemistry Department of the University of Birmingham, England (7). The starch was character-

of the "starches" obtained by these investigators with our preparations is not possible, since the same criteria of characterization have not been employed. However, on the basis of existing data it appears quite probable that the leaf "starches" isolated by Spoehr and Milner are similar to the polysaccharides described in this and a previous communication (1).

EXPERIMENTAL

All analytical constants and rotations are reported on the ash- and moisture-free basis.

Preparation of Crude Starch Polysaccharide—The leaves from 8 year-old apple trees (same variety and vegetative condition) were collected on a sunny afternoon during the latter part of August, 1933. They were first subjected to a temperature of 115° under 15 pounds pressure for 10 minutes to inactivate the enzymes, and then dried rapidly in a strong current of air at 65° (8, 9). The dried tissue was ground to pass a 120 mesh sieve, and exhaustively extracted first with acetone and then with a mixture of benzene and ethanol (ratio 2:1). 6 kilos of the air-dried extracted tissue were refluxed for 45 minutes with 24 liters of 85 per cent ethanol containing 0.75 per cent nitric acid. The solids were recovered by centrifugation and again treated with the acid-alcohol reagent. This operation was repeated until two successive extractions produced solutions of the same color. The residue was then suspended in 20 liters of cold water, thoroughly stirred for 15 minutes, and centrifuged. The insoluble portion was washed with water until the washings were neutral to litmus. After spinning to partial dryness, the mass was extracted for 20 minutes with 30 liters of hot water in a steam-jacketed vessel to remove the starch polysaccharide. The insoluble leaf residue was separated from the hot solution by centrifugation and again extracted for 20 minutes with 30 liters of boiling water. The first and second aqueous extracts were combined and concentrated *in vacuo* (15 to 20 mm.) at 30° to 2 liters. The concentrate was filtered through

ized as an amorphous white substance, soluble in cold water, and yielded (like β -amylase) the theoretical quantity of maltose with barley diastase. Dr. Beazeley has informed us in a private communication that a complete report of this work has so far not been published.

an asbestos mat and the light yellow filtrate poured into 4 volumes of 95 per cent ethanol. The precipitated material was allowed to settle and the major portion of the supernatant liquid was removed. The remaining gelatinous suspension was made up to 5 liters with a 1:1 acetone-ethanol mixture, and allowed to stand for several days. After removing most of the acetone-ethanol solution by decantation, the starch polysaccharide slurry was diluted with acetone, collected on a suction filter, pressed dry under a rubber dam, and finally washed with dry acetone. After the cake was disintegrated, the product was dried *in vacuo* over calcium chloride for 4 days. After it was ground to a fine powder, it contained 11 per cent moisture. Yield, 100 gm.

Preliminary Purification of Starch Polysaccharide—80 gm. of the crude polysaccharide were shaken with 2 liters of cold water for 2 hours. The resulting dispersion was centrifuged, yielding a cloudy supernatant liquid and a mucilaginous residue. The latter was suspended in 2 liters of water, centrifuged, and the resulting aqueous extracts combined. This turbid solution was passed through a Berkefeld type N filter, and the polysaccharide recovered from the clear but slightly yellow filtrate by precipitation with ethanol and acetone. Yield, 25 gm. 20 gm. of the above product were dispersed in 1 liter of cold water, the resulting solution filtered through a type N Berkefeld filter, the polysaccharide recovered from the filtrate as above, and dried *in vacuo* over calcium chloride. Yield, 15 gm. The polysaccharide thus obtained contained 9 per cent moisture and 2.7 per cent ash. It was soluble in water, yielding a slightly opalescent solution. This aqueous solution on treatment with the iodine-potassium iodide reagent produced a blue color which was discharged on heating and which reappeared on cooling. The blue starch iodide color was also discharged by the addition of aqueous silver nitrate (10). The application of Small's test for erythrodextrin (3) and Bial's reaction for pentosan (4) indicated that these substances were still present in the polysaccharide which possessed the following constants.

Specific Rotation— $[\alpha]_D^{20} = +172^\circ \pm 2^\circ$ (in water, $c = 0.9$ per cent).

Relative Viscosity— $t/t_0 = 1.12$ at 35° (in water, $c = 1$ per cent).

Analysis— $(C_6H_{10}O_5)_x$. Calculated. C 44.4, H 6.1

(Micro-Pregl) Found. " 44.0, " 5.9

Final Purification of Starch Polysaccharide—3 gm. of the partially purified polysaccharide were dissolved in 200 cc. of warm water, to which 20 cc. of an aqueous solution containing 4 per cent iodine and 6 per cent potassium iodide were added. The starch-iodide complex was then flocculated by the addition of an equal volume of saturated ammonium sulfate (3). The resulting suspension was centrifuged, the supernatant liquid decanted, and the residue taken up in 100 cc. of water. An equal volume of saturated ammonium sulfate was then added, and the process repeated until the supernatant liquid was colorless after centrifugation. After the last washing was decanted, the precipitate was drained and suspended in 100 cc. of water. 0.1 per cent silver nitrate was then added until the blue color was discharged. The solution was filtered (Berkefeld type N) and the filtrate made up to 200 cc. Ammonia was determined on an aliquot portion and to the remainder of the solution sufficient barium acetate was added to react quantitatively with the ammonium sulfate present. The barium sulfate was removed by filtration (Berkefeld type N) and the filtrate poured with stirring into 3 liters of a 1:1 acetone-ethanol solution. The precipitate was collected by centrifugation and taken up in 50 cc. of warm water. This solution was filtered through an asbestos mat and the polysaccharide recovered from the filtrate as above. Yield, 1.6 gm. Aqueous solutions of this polysaccharide on treatment with iodine-potassium iodide produced the pure blue color characteristic of β -amylose. Erythro-dextrins and pentosans were definitely absent. The following constants were observed.

Specific Rotation— $[\alpha]_D^{20} = +185^\circ \pm 2^\circ$ (in water, $c = 0.7$ per cent).

Analysis— $(C_6H_{10}O_5)_x$. Calculated. C 44.4, H 6.1

Found. " 44.6, " 5.95

Acid Hydrolysis of Starch Polysaccharide. Complete Hydrolysis—Samples of the partially purified polysaccharide, the purified polysaccharide, and potato starch were hydrolyzed with 2.5 per cent sulfuric acid by refluxing for 3.5 hours. After cooling, the hydrolysates were neutralized, made to volume, and utilized for the determination of reducing sugars and specific rotation. The values so obtained are as follows:

$[\alpha]_D^{30}$	Glucose yield
degrees	per cent
+65.0	90.0
+62.0	98.0
+62.0	98.1

2.0 gm. of the partially purified polysaccharide were hydrolyzed as above and the acid hydrolysate made up to 100 cc. 50 cc. of this solution were withdrawn, adjusted to pH 4.0, and inoculated with a pure culture of *Saccharomyces cerevisiae* after the addition of an equal volume of double strength yeast water. Upon incubation for 5 days at room temperature, the solution had no perceptible action on boiling Fehling's solution, thereby indicating that the polysaccharide in question was composed of units of fermentable monosaccharides, e.g. *d*-glucose, *d*-fructose, or *d*-mannose. The remainder of the acid hydrolysate was neutralized with barium carbonate, the precipitated barium sulfate removed, and the solution concentrated *in vacuo* to a small volume. The application of Seliwanoff's reaction (4), with a small portion of the concentrate, established the absence of *d*-fructose. On treating the remainder of the concentrate with phenylhydrazine in the presence of acetic acid, the absence of *d*-mannose was indicated by the non-appearance of its insoluble hydrazone and finally from this reaction mixture *d*-glucosazone was isolated in good yields. The constants of this preparation are given below.

Melting Point—The compound melted at 205–206° with decomposition.

Analysis— $C_{18}H_{28}O_4N_4$. Calculated, N 15.64; found, N 15.50 (Pregl).

Kinetics of Acid Hydrolysis—The kinetics of the acid hydrolysis of the partially purified polysaccharide were studied at 80° and 90° in a solution which was 5 N in sulfuric acid and approximately 1 per cent in starch content. The rate of hydrolysis was followed by determining the liberated reducing groups with the volumetric method of Benedict (11). The data so obtained were reduced to a first order equation whose constants are as follows:²

² In this and the previous communication (1) we have employed this method as a means of obtaining numerical values solely for purposes of characterization. In every case the constants presented are average values. It is not implied that the first order equation describes exactly the course of the acid hydrolysis of starch polysaccharides (12).

Kinetics of 5 N H₂SO₄ Hydrolysis of Apple Leaf Polysaccharide

$K_{90} \text{ min.}^{-1}$	$K_{90} \text{ min.}^{-1}$	K_{90}/K_{80}
$\times 10^{-1}$	$\times 10^{-1}$	
2.2	7.2	3.27
1.5	5.1	3.40
	4.5	

Enzymatic Hydrolysis of Starch Polysaccharide. Isolation of Maltose—1 gm. of the partially purified polysaccharide was dissolved in 50 cc. of water. This solution was buffered to pH 4.5 by the addition of 5 cc. of 0.1 M sodium acetate-acetic acid. 10 cc. of a solution of barley malt diastase (1) were added, and the reaction mixture maintained at 37° for 48 hours. The digest was evaporated to dryness at 30°, taken up in hot 70 per cent ethanol, filtered, and the filtrate concentrated under diminished pressure to 10 cc. The concentrate was then treated with phenylhydrazine and maltosazone isolated in the usual manner.

Melting Point—The derivative melted at 205–206° with decomposition.

Analysis—C₂₄H₃₁O₉N₄. Calculated, N 10.80; found, N 10.94

Kinetics of the Enzymatic Hydrolysis—The kinetics of the enzymatic hydrolysis of the partially purified leaf starch polysaccharide were studied at 37° in an aqueous solution buffered to pH 4.5. The hydrolysis was conducted as previously described, with barley malt diastase (1). Soluble potato starch was employed as a control. Calculated as before (1), the following constants were obtained: leaf starch polysaccharide $K_{37} = 1.2 \times 10^{-3} \text{ min.}^{-1}$; potato starch polysaccharide $K_{37} = 1.5 \times 10^{-3} \text{ min.}^{-1}$.

Determination of "Chromic Period" (13)—2 per cent solutions of the purified leaf starch polysaccharide and a soluble potato starch polysaccharide were prepared. 0.25 cc. of starch solution, 0.05 cc. of 0.1 M sodium acetate-acetic acid, and 0.1 cc. of enzyme solution were introduced into a small test-tube and the volume adjusted to 1 cc. The solution was maintained at 37°, and at intervals a drop was removed and tested against a drop of iodine-potassium iodide reagent. The time required for the disappearance of the last trace of blue color was recorded as the "chromic period." Since this property is greatly dependent upon the

enzyme concentration as well as the substrate, the values so obtained were reduced to ratios, with the chromic period of the potato starch polysaccharide taken as unity. This procedure offers a comparison of substrates independent of enzyme variation. The values thus found are given in Table I.

TABLE I
Relative "Chromic Periods"

Enzyme	Dilution	Relative "chromic period" (period for potato starch = 1)
Barley malt diastase	0	1.0
	1:10	1.0
" diastase	0	0.9
Taka-diastase	0	1.0
	1:10	0.9

SUMMARY

A starch polysaccharide has been isolated from the leaf tissue of the apple tree (*Malus malus*). This polysaccharide was found to be a polyglucosan, similar, if not identical, in structure to the β -amylose component of common cereal and tuber starches and to the starch polysaccharide previously isolated from the woody tissue of the apple tree.

We are indebted to Mr. Robert Harrower and Dr. Eugene Schoeffel for assistance with the analytical determinations and to Professor R. H. Roberts of the Horticulture Department for his cooperation and counsel.

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THE FORMATION OF TAURINE BY THE DECARBOXYLATION OF CYSTEIC ACID*

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The transformation of cystine into taurine *in vitro* was first reported by Friedmann in 1903 (1). This investigator converted cystine to cysteic acid by oxidation with bromine, and subsequently decarboxylated the cysteic acid by heating the latter compound in aqueous solution in a sealed tube at a specified temperature. These reactions have received considerable attention, because on them has rested in part the assumption that the taurine of the bile salts has its origin in dietary cystine. However, subsequent investigators have had difficulty repeating Friedmann's synthesis. It is the decarboxylation reaction which does not, apparently, proceed as described by Friedmann. Lewis and Lewis (2), in a footnote, have mentioned the only successful decarboxylation of cysteic acid in the literature, other than that of Friedmann.¹ Gortner and Hoffman (3) made several unsuccessful attempts to conduct this reaction and suggested that inasmuch as Friedmann's cysteic acid was prepared from the copper salt, while theirs was not, "It may be that copper catalyzes the reaction." Since Gortner and Hoffman did not test this possibility, one of the present authors (W.), in collaboration with

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¹ In a private communication, Professor Howard B. Lewis has informed us that the single decarboxylation of cysteic acid reported from his laboratory was one of approximately twenty attempts and subsequent efforts to repeat the reaction were unsuccessful. A gas-heated bomb furnace was used.

Dr. H. B. Vickery, has attempted to repeat Friedmann's synthesis exactly as described by the latter investigator. However, efforts to decarboxylate cysteic acid, prepared either from the copper salt or by direct crystallization, were unsuccessful.

Recently, the decarboxylation of cysteic acid has again been studied in some detail. The evidence which has been obtained indicates that the reaction proceeds *only within a limited temperature range*, and unless the latter factor is carefully regulated, the results are likely to be negative. By the use of a gas-heated furnace and accurate control of the temperature *inside* the iron pipe in which the bomb tube rested, it was found possible to prepare taurine repeatedly by the decarboxylation of cysteic acid as described by Friedmann. The identity of the product was established by analysis, by conversion into its N-phenylcarbamyld derivative and analysis of this compound, and finally by comparison with a sample of synthetic taurine prepared by Cortese (4) by the interaction of β -aminoethyl bromide hydrobromide and sodium sulfite.² It might also be noted that the decomposition point of taurine recorded in the present investigation is approximately 85° higher than that given by handbooks (*cf.* (5)). This error in the literature may have possibly led investigators in certain instances to overlook the fact that they had obtained taurine from reactions carried out in the effort to repeat Friedmann's work.

EXPERIMENTAL

Preparation of Cysteic Acid—Cysteic acid was prepared from L-cystine by oxidation with bromine according to Friedmann (1). However, purification through the copper salt was omitted. The crude product was crystallized directly, and recrystallized from dilute alcohol. It was dried overnight at 105° for analysis. 30 gm. of cystine gave 19.3 gm. of cysteic acid. The product melted at 267° with decomposition.³

Analysis—(Kjeldahl)

$C_3H_7O_4NS$. Calculated, N 8.28; found, N 8.30, 8.36

² The authors wish to thank Dr. Frank Cortese for a generous sample of his synthetic taurine.

³ All melting points are corrected and were obtained by the capillary tube method, with the one exception noted in the text.

Decarboxylation of Cystic Acid—2 gm. of cystic acid were dissolved in 15 cc. of water and the solution transferred to a Pyrex bomb tube. The tube was sealed and placed in an iron pipe which formed the inner core of a gas-heated bomb furnace. The temperature of the pipe in which the bomb tube rested was brought to 235° during a period of 3 hours. The temperature was then kept between 235–240° for a further 2 hour heating period. The furnace was allowed to cool overnight and the bomb tube opened. A strong mercaptan odor was evident. The solution was dark brown in color, with a green fluorescence. The contents of the tube were filtered, and the filtrate and washings concentrated on the steam bath *in vacuo* until a dark, crystalline mass began to separate. The material in the flask was redissolved in a minimum quantity of hot water, decolorized, filtered, and the filtrate and washings again concentrated *in vacuo* until crystals appeared. After cooling overnight in the ice box, the slightly colored needles which had separated were filtered and washed with cold, 50 per cent ethyl alcohol. The dry material weighed 0.86 gm. From the mother liquor a second crop weighing 0.36 gm. was obtained by the addition of 2 volumes of absolute ethyl alcohol.

This experiment is typical of eight decarboxylations which have been conducted. A uniformity of results was observed in all experiments. For analysis, 3.25 gm. of the crude product were recrystallized twice from hot water. The material has a tendency to crystallize from concentrated solutions in very large, prismatic needles. The dried product weighed 2.7 gm. It had the following composition.⁴

$C_2H_7O_3NS$.	Calculated.	C 19.20,	H 5.60,	N 11.20,	S 25.60
	Found.	" 19.57,	" 5.72,	" 11.03,	" 25.53

The taurine melted with decomposition at 327–328°. Through the kindness of Professor V. du Vigneaud, the melting point of our preparation was determined in his laboratory on the Dennis

⁴ The microanalyses for carbon, hydrogen, and sulfur reported in this paper were conducted by Dr. G. Weiler, Oxford, England. The micro-Dumas nitrogen determination was carried out in the laboratory of Dr. H. T. Clarke, Columbia University.

bar; the melting point was found to be $328^{\circ} \pm 1^{\circ}$. Cortese (4) reports that his synthetic taurine decomposed at $300\text{--}305^{\circ}$. However, in our laboratory his product has consistently melted with decomposition at $321\text{--}324^{\circ}$. The melting point obtained with a mixture of our product and that of Cortese was 327° with decomposition. There is some variation in decomposition point with the rate of heating of the bath, but this variation is not significantly great. Essentially the same data for the temperature of decomposition are obtained when the decomposition point is approached at the rate of 2° , 3° , or 5° per minute. It has also been possible to check the decomposition points of several other specimens of taurine. Professor H. B. Lewis generously furnished a sample of taurine which was prepared in the one successful decarboxylation conducted in his laboratory. This material decomposed at $314\text{--}316^{\circ}$. It was recrystallized from hot water. The purified substance decomposed at 323° . Taurine synthesized according to Marvel, Bailey, and Sparberg (6), and given to us by Dr. J. White, decomposed at 322° . Taurine prepared from ox bile, in the laboratory of Professor Lewis, melted with decomposition at 326° . Professor C. L. A. Schmidt has kindly supplied a sample of taurine prepared in his laboratory from the abalone. After recrystallization, the specimen melted at 321° . It would seem, therefore, that the melting point of taurine recorded in the literature has been erroneous, and furthermore that there is no variation in decomposition point of taurine when the latter compound is obtained either by synthetic procedures or from natural sources.

N-Phenylcarbamyltaurine was synthesized, according to Schöberl (7), from the taurine prepared in the present investigation. Microanalysis showed it to have the following composition.

$C_9H_{12}O_4N_2S$.	Calculated.	C 44.26, H 4.92
	Found.	" 44.31, " 5.08

The derivative melted with decomposition at 197° . Furthermore, a sample of N-phenylcarbamyltaurine prepared from the synthetic taurine supplied by Dr. Cortese also melted at 197° . There was no depression in melting point of a mixture of the derivatives prepared from the two samples of taurine.

SUMMARY

Conditions have been described for the successful repetition of Friedmann's transformation of cystine into taurine. The melting point of taurine recorded in the literature has been demonstrated to be erroneous and a considerably higher decomposition point has been definitely established.

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STUDIES OF MULTIVALENT AMINO ACIDS AND PEPTIDES

VII. DERIVATIVES OF *dl*- α -AMINOTRICARBALLYLIC ACID

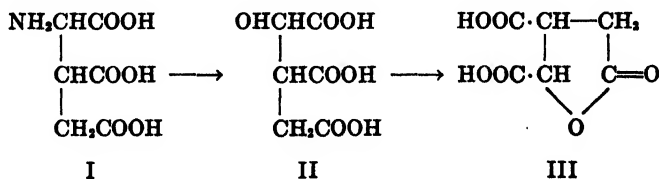
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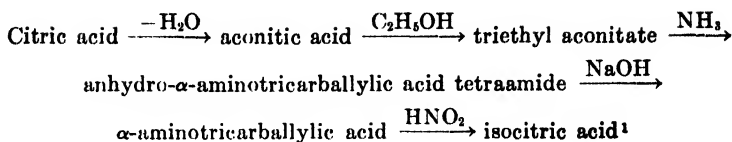
(Received for publication, August 17, 1936)

dl- α -Aminotricarballylic acid is the first amino acid to be prepared containing three carboxyl groups (5). The electrochemical properties of this substance have been previously described (6). In structure it is very similar to glutamic acid, differing from the latter in possessing a β -carboxyl group. It would seem of interest to synthesize certain derivatives of aminotricarballylic acid, on the one hand to confirm its constitution and on the other to compare such derivatives with those of glutamic acid.

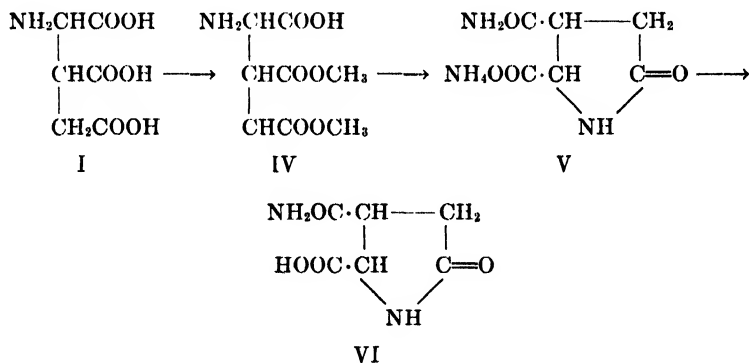
Accordingly, the amino acid (I) was first deaminated with nitrous acid, yielding isocitric acid (II) from which the corresponding crystalline lactone (III) was obtained.



Inasmuch as the amino acid itself was obtained from citric acid, the entire series of reactions describes the preparation of *dl*-isocitric acid (α -hydroxytricarballylic acid) from citric acid (β -hydroxytricarballylic acid).



Glutamic acid, particularly in the ester form, passes readily into the corresponding pyrrolidone- α -carboxylic acid (3). This behavior is paralleled in the case of α -aminotricarballylic acid. On partial esterification of the latter substance (I), the β, γ two-thirds diester is obtained (IV) in analogy with the γ half ester of glutamic acid (1, 7). In concentrated aqueous ammonia, the two-thirds ester is converted by ring closure into the pyrrolidone- α -ammonium carboxylate β acid amide (V). From the silver salt of the latter, pyrrolidone- α -carboxylic acid β acid amide (VI) was prepared.



EXPERIMENTAL

dl-Isocitric Acid (II)—2 gm. of pure, crystalline α -aminotricarballylic acid were dissolved in 60 cc. of 1 N HCl, chilled, and 6.2 gm. of AgNO₂ added with vigorous shaking over a period of 30 minutes. The mixture was then allowed to stand for 24 hours, the

¹ Isocitric acid was not believed to occur in nature until 1925 when Nelson isolated it from the juice of blackberries (8). As the lactone, it apparently forms about five-sixths of the total acids of this fruit and may be more wide-spread in nature than has been supposed. Bruce (2) has improved the method of isolation of this substance from blackberries. The only recorded synthesis of isocitric acid is that by Fittig, who obtained it by the decomposition of trichloromethylparaconic acid with baryta (4).

AgCl filtered off and washed, and H_2S in excess passed through the filtrate. The filtered solution (through norit) was taken to a syrup *in vacuo*, transferred to a vacuum desiccator, and finally dried over P_2O_5 and KOH. After 2 days the syrup sets to a solid mass, most probably a mixture of isocitric acid and its lactone. The mass was dissolved in about 20 cc. of water and saturated baryta solution added until the mixture was just alkaline to litmus. The mixture was then boiled for 4 hours under a reflux provided with a soda-lime inlet, then filtered rapidly, and the residue washed repeatedly with hot water until Cl-free. The yield of the barium salt was almost quantitative. It was dried *in vacuo* at 100° over P_2O_5 for several hours and analyzed for Ba.

$\text{C}_{12}\text{H}_{10}\text{O}_4\text{Ba}_2$ (790.2). Calculated, Ba 52.1; found, Ba 51.5

Conversion of Isocitric Acid into Its Lactone (III)—3 gm. of the barium isocitrate were suspended in dilute HCl, heated, and the Ba quantitatively removed with dilute H_2SO_4 . The filtrate was evaporated *in vacuo* to a syrup. After several days standing over P_2O_5 the syrup solidified. It was extracted with hot acetone and filtered off from a small amount of inorganic impurity. The acetone was evaporated and the residue heated *in vacuo* at 100° for 3 hours. By this means any isocitric acid present was converted into the corresponding butyrolactone dicarboxylic acid. The latter was then dissolved in a relatively large amount of hot, dry ethyl acetate, filtered, and the filtrate evaporated to a small bulk. The lactone was then crystallized by the careful addition of dry petroleum ether. Long prisms formed. The melting point was 153° , identical with that of the lactone prepared from the optically active acid (2). Yield 0.6 gm.

$\text{C}_8\text{H}_6\text{O}_6$ (174.0). Calculated, C 41.4, H 3.4; found, C 41.4, H 3.3

α -Aminotricarballylic Acid β,γ -Dimethyl Ester (IV)—4 gm. of the amino acid were suspended in 100 cc. of absolute methanol containing 5 gm. of HCl gas. On shaking, the acid went immediately into solution. After standing half an hour at room temperature, the solution was taken down to a syrup *in vacuo*, the syrup taken up in a little methanol, and precipitated by addition of an excess of dry ether. The supernatant fluid was decanted and the syrup dried *in vacuo*. It set to a foamy solid. The latter was

dissolved in 50 cc. of water and treated with a slight excess of Ag_2O . After filtration, hydrogen sulfide was bubbled through the filtrate and the silver sulfide filtered off and washed. The solution was now concentrated *in vacuo* until crystallization began. The entire concentrate, taken up in a little hot water, was treated with a large excess of acetone. Crystallization of the two-thirds ester began rapidly in clusters of thin prisms. Yield 0.9 gm. M.p. sharp at 165° . The substance was very soluble in water, slightly soluble in hot methanol, insoluble in all other organic solvents. It gave a positive ninhydrin reaction.

$\text{C}_8\text{H}_{13}\text{O}_5\text{N}$ (219.1). Calculated. C 43.9, H 5.9, N 6.4
Found. " 43.8, " 6.0, " 6.5

Pyrollidone- α -Ammonium Carboxylate β Acid Amide (V)—0.6 gm. of the two-thirds ester was dissolved in 8 cc. of concentrated ammonia (28 per cent) in water and the solution allowed to stand at room temperature for 5 days. After filtering, the solution was dried *in vacuo* over H_2SO_4 . The residue sets to a solid mass of prisms. It is rubbed up with acetone and filtered. Yield 0.5 gm. M.p. sharp at 214° . On warming with alkali, copious fumes of NH_3 are evolved. The molecule of crystal water is held firmly, even after the substance is heated for several hours at 100° at 20 mm.

$\text{C}_6\text{H}_{11}\text{O}_4\text{N}_3 + 1\text{H}_2\text{O}$ (207.1). Calculated. C 34.8, H 6.3, N 20.3
Found. " 35.2, " 6.1, " 19.9

Pyrollidone- α -Carboxylic Acid β Acid Amide (VI)—0.35 gm. of the ammonium salt in 3 cc. of water was treated with an aqueous solution of 0.5 gm. of AgNO_3 . The dense flocculent precipitate of the silver salt was filtered off and washed with a little cold water. After decomposition with H_2S , the filtrate on evaporation yielded a colorless, viscous syrup which crystallized after several days into prisms. The substance was rubbed up with acetone and dried. Yield 0.1 gm. M.p. 178° .

$\text{C}_6\text{H}_9\text{O}_4\text{N}_2$ (172). Calculated. C 41.8, H 4.7, N 16.2
Found. " 41.8, " 4.8, " 15.9

SUMMARY

1. *dl*- α -Aminotricarballylic acid was converted by deamination into *dl*-isocitric acid from which the crystalline lactone was derived.

2. A synthesis of isocitric acid from citric acid is described.

3. The β,γ two-thirds dimethyl ester of α -aminotricarballylic acid was prepared (m.p. 165°) from which the corresponding pyrrolidone- α -ammonium carboxylate β acid amide (m.p. 214°) was obtained by ring closure. Decomposition of the silver salt of the latter yielded pyrrolidone- α -carboxylic acid β acid amide (m.p. 178°).

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A SYNTHESIS OF GLUTATHIONE

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The reduction of dicarbobenzoxy-cystinyldiglycine and of S-benzylcysteinylglycine to cysteinylglycine by metallic sodium in liquid ammonia was presented in the recent work on crystalline cystinyldiglycine by Loring and du Vigneaud (1). The effectiveness of these two reactions impressed us with their potentialities as possible key reactions for a new synthesis of glutathione; if N-carbobenzoxy- γ -glutamyl-S-benzylcysteinylglycine could be prepared, its reduction with sodium in liquid ammonia would be expected to yield glutathione.

This approach to glutathione would have a particular advantage in that the sulfhydryl group would be covered by a benzyl radical up to the final step and thus, during the course of the various reactions that might be employed up to this point, the likelihood of partial oxidation of the sulfhydryl group and its attendant difficulties would be eliminated. Even where the disulfide form might be used in any of the steps, the benzylthio ether linkage would still have the advantage of being more stable. The removal of the benzyl group would not involve an extra step, since it would be accomplished during the same reaction as that employed for the cleavage of the carbobenzoxy group.

We have undertaken the synthesis of glutathione along these lines and a method has been worked out which we feel offers excellent possibilities as a preparative procedure for obtaining this physiologically significant tripeptide. The yields throughout the synthesis have been encouragingly high and the reactions involved are such that one should be able to carry them out on a fairly large scale. The successful outcome of the synthesis has also

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afforded a confirmation of the synthetic proof of structure of glutathione, which was recently accomplished by Harington and Mead (2). The method of synthesis devised by these authors, although sufficing for structural proof, gave a very small yield.

In the present work, α -methyl-N-carbobenzoxy- γ -glutamyl-S-benzylcysteinylglycine methyl ester was prepared by the condensation of α -methyl-N-carbobenzoxyglutamyl chloride, which had been used by Harington and Mead (2), with S-benzylcysteinylglycine methyl ester in chloroform. 3 moles of the latter were employed in the reaction to 1 of the acid chloride. By using an excess of the basic ester the use of a foreign condensing agent was obviated. Furthermore, the use of an excess of the ester rather than an excess of the acid chloride seemed preferable to us, since the ester was very readily recoverable. After the condensation reaction was completed, the chloroform solution was extracted with dilute HCl. The excess S-benzylcysteinylglycine methyl ester was recovered from this extract and was used in subsequent reactions. The chloroform layer was evaporated and the condensation product so obtained was saponified. The resulting N-carbobenzoxy- γ -glutamyl-S-benzylcysteinylglycine was then dissolved in liquid ammonia and reduced by metallic sodium. After spontaneous evaporation of the ammonia, the residue was dissolved in sufficient 0.5 N H_2SO_4 to make the solution acid to litmus and the glutathione was precipitated as the mercury compound by the addition of mercuric sulfate. The mercury salt was decomposed with H_2S and the glutathione was reprecipitated with cuprous oxide. The cuprous glutathione showed the characteristic sheen mentioned by Hopkins (3). After the copper was removed with H_2S , the aqueous filtrate from the cuprous sulfide was evaporated to a small volume and the glutathione was crystallized in the usual manner.

The method of obtaining the free ester of S-benzylcysteinylglycine is worth calling attention to as it may find wider application. The free ester was prepared by the addition of an excess of diethylamine to a chloroform solution of the ester hydrochloride, followed by the addition of ether. The diethylamine hydrochloride was precipitated, leaving the free amino ester in solution. We had encountered much difficulty with diketopiperazine formation in attempts to make the free ester by other

procedures. In the diethylamine procedure, this tendency towards diketopiperazine formation was greatly minimized. The diethylamine, of course, can be readily recovered from the hydrochloride.

The glutathione obtained by the synthesis had the same crystalline form as that of an authentic sample isolated from natural sources. The compounds melted at the same temperature, namely 190° , and a mixture of the synthetic and naturally occurring compounds gave no depression of melting point. The compounds further gave the same specific rotation of $[\alpha]_D^{27} = -21.3^{\circ}$ for a 2 per cent solution in water.

EXPERIMENTAL

Preparation of N-Carbobenzoxy- γ -Glutamyl-S-Benzylcysteinylglycine—The S-benzylcysteinylglycine was prepared from dicarbobenzoxycystinyldiglycine by reduction in liquid ammonia with sodium and subsequent addition of benzyl chloride to the liquid ammonia solution of the reduced dipeptide, according to the directions of Loring and du Vigneaud (1). The ester hydrochloride was prepared by suspending 30 gm. of the S-benzylcysteinylglycine in 150 cc. of anhydrous methyl alcohol and saturating with dry HCl the mixture cooled in an ice-salt bath. As the HCl was being introduced the S-benzylcysteinylglycine readily passed into solution. The excess of HCl was removed with the aid of a water pump and the solution was concentrated *in vacuo* without application of heat. The syrupy residue was dissolved in 150 cc. of anhydrous methyl alcohol. The solution again was saturated at 0° with HCl and was concentrated as before. In order to remove excess HCl and methyl alcohol from the resulting residue, it was dissolved and reevaporated once with methyl alcohol and then three times with anhydrous chloroform.

The ester hydrochloride was dissolved in 60 cc. of purified chloroform. The solution was cooled in a solid CO_2 -trichloroethylene bath and to it were added 23 cc. of diethylamine. The solution was allowed to warm to about 0° , and 250 cc. of anhydrous ether were added with shaking. The crystalline diethylamine hydrochloride was filtered and was washed several times with ether. The clear, pale yellow filtrate was concentrated at a temperature below 0° . In order to remove excess diethyl-

amine from the syrupy residue, it was twice taken up in cold chloroform and was concentrated after each addition. The final product was dissolved in 75 cc. of chloroform and was stored temporarily in a solid CO₂-trichloroethylene bath while the acid chloride with which it was to be condensed was being prepared.

The α -methyl-N-carbobenzoxyglutamate was prepared according to the directions of Harington and Mead (2) who utilized the observation of Melville (4) that treatment of carbobenzoxyglutamic anhydride with sodium ethylate yielded preponderantly the α ester and that the γ -chloride of the latter could then be prepared. 11 gm. of α -methyl-N-carbobenzoxyglutamate were dissolved in 35 cc. of anhydrous ether. The solution was cooled in an ice-salt bath and 10 gm. of finely pulverized PCl₅ were added in one portion. After the mixture was shaken for 20 minutes, the supernatant solution was decanted from a small amount of unchanged PCl₅ and was concentrated *in vacuo* at 0°. When the solution began to acquire a syrupy consistency, the distillation was stopped. The acid chloride was precipitated by adding 50 cc. of ice-cold anhydrous petroleum ether (boiling point, 70°). After the mixture was thoroughly shaken to dissolve the POCl₃, the petroleum ether layer was decanted. The acid chloride again was washed by shaking vigorously with 50 cc. of cold petroleum ether. The process was repeated a third time. The acid chloride was next dissolved in 50 cc. of chloroform which had been precooled in a solid CO₂ bath and the solution was used immediately for the next reaction. Since the compound has been found to decompose very rapidly, too much emphasis cannot be placed on the speed with which the latter steps should be carried out.

The chloroform solution of the acid chloride was poured immediately into the cold solution of the methyl ester of S-benzylcysteinylglycine which had just been prepared as indicated above. The mixture was allowed to stand at room temperature for 3 hours. The clear, yellow solution was extracted ten times with 25 cc. portions of 0.1 N HCl, then four times with water. The chloroform layer was dried over anhydrous sodium sulfate and was evaporated *in vacuo* to a dry solid residue. The crude condensation product was removed from the flask with the aid of a little ethyl acetate and precipitation of the material which had

dissolved in the ethyl acetate was accomplished by the addition of several volumes of petroleum ether. The material was filtered, washed with petroleum ether, and was used for the saponification.

The excess ester hydrochloride used in the condensation reaction was recovered to the extent of 88 per cent. The pH of the combined acid and water extracts was adjusted to approximately 4.0 and the solution was concentrated to a syrup. The residue was taken up in alcohol and the solution was evaporated. The compound was dissolved in anhydrous chloroform, was filtered to remove the NaCl, and the filtrate was concentrated. The residue was dissolved again in chloroform and was reevaporated. After this treatment the material was suitable for use again in another condensation reaction.

For the saponification of the condensation product, the method used by Harington and Mead for the saponification of α -methyl-N-carbobenzoxy- γ -glutamylcysteinylglycine ethyl ester (2) was found to be applicable. 20 gm. of the crude α -methyl-N-carbobenzoxy- γ -glutamyl-S-benzylcysteinylglycine methyl ester were dissolved in 200 cc. of dioxane. Sufficient alkali was added to make the solution alkaline to litmus. After this point was reached, there was gradually added to the solution a 20 per cent excess of the theoretical amount of N NaOH required to saponify the ester. The reaction mixture was allowed to stand at room temperature for 1 hour, at the end of which time it was neutralized with an amount of N HCl equivalent to the NaOH added. The solution was decolorized by allowing it to stand with carbex E for 2 hours at room temperature. The filtrate from the carbex E was concentrated *in vacuo* without application of heat. The semisolid residue was taken up in ethyl acetate. The acidic product was extracted from the ethyl acetate solution with saturated NaHCO₃ solution. After the NaHCO₃ extract was washed with ether, it was carefully acidified. At a pH of about 5.0 a small amount of amorphous impurity separated. This was filtered and the filtrate was further acidified. The compound came out of solution as an oil which hardened on standing. The solidified material was broken up, filtered, washed with water, and dried. 14 gm. of the crude material suitable for the reduction step were obtained. Attempts at crystallization have so

far proved unsuccessful. The compound, after solution in absolute alcohol and precipitation with water, was analyzed for sulfur and nitrogen. The values of 6.32 per cent for sulfur and 8.03 per cent for nitrogen were obtained in comparison with the theoretical values of 6.00 and 7.91 respectively based on the empirical formula of $C_{25}H_{29}O_8N_3S$.

On the basis of the α -methyl-N-carbobenzoxyglutamate used to prepare the acid chloride employed in the condensation, the yield of 14 gm. of saponified product represents 72 per cent of the theoretical yield. On the basis of the S-benzylcysteinylglycine used, allowing for the amount of excess ester actually recovered from the condensation reaction, the yield was 57 per cent. This represents the over-all yield through the esterification of S-benzylcysteinylglycine, freeing of the ester, the condensation with the acid chloride, and the saponification. It might also be mentioned that the S-benzylcysteinylglycine was obtained in a 50 per cent yield based on the original amount of cystine started with and the α -methyl-N-carbobenzoxyglutamate was obtained in a 63 per cent yield based on the glutamic acid used.

Reduction of N-Carbobenzoxy- γ -Glutamyl-S-Benzylcysteinylglycine—7 gm. of crude N-carbobenzoxy- γ -glutamyl-S-benzylcysteinylglycine were dissolved in 100 cc. of dry liquid ammonia in a 3-neck 200 cc. flask equipped with a mercury seal stirrer and a soda-lime tube. 1.5 gm. of sodium were added gradually in small pieces and the reaction mixture was stirred at a moderate rate. 4.3 gm. of ammonium sulfate, which were equivalent to the amount of sodium used, were added. After the solution was stirred for about 15 minutes longer, the ammonia was allowed to evaporate spontaneously. The last portions of ammonia were removed in a vacuum desiccator over H_2SO_4 . Another 7 gm. of the saponified condensation product were reduced with sodium in a manner similar to that described above and the combined solid residues from the two reactions were dissolved in 250 cc. of cold 0.5 N H_2SO_4 . The solution which resulted was acid to litmus. The mercaptide was first precipitated with a modified Hopkins' mercuric sulfate reagent (5). The mercury salt was washed with water several times at the centrifuge. Then it was suspended in about 50 cc. of water and decomposed with H_2S . The mercuric sulfide was separated by centrifugation and was washed

several times with water. The washings were combined with the main solution. After the removal of excess H_2S by bubbling hydrogen through the solution, an equal volume of $\text{N H}_2\text{SO}_4$ was added. The solution was heated to 40° and was treated with cuprous oxide according to Hopkins' procedure (3). A crystalline precipitate possessing the sheen characteristic of cuprous glutathione separated. The cuprous salt was washed with oxygen-free water until it was free of H_2SO_4 . It was then suspended in about 50 cc. of water and decomposed with H_2S . The cuprous sulfide was filtered and the excess H_2S was removed from the filtrate by passing a stream of hydrogen through it. The solution then was evaporated rapidly in a vacuum desiccator over P_2O_5 . The glassy residue was dissolved in sufficient water to give a heavy syrup. After a small amount of alcohol was mixed with the syrup, the solution was seeded and was placed in a desiccator over solid NaOH and P_2O_5 . The desiccator was filled with hydrogen and was placed in the refrigerator. Crystallization was allowed to proceed for 3 days, at the end of which time the material was taken up with the aid of a little 75 per cent alcohol and filtered. The product was washed first with 75 per cent alcohol and finally with 95 per cent alcohol and then dried. 1.3 gm. of beautifully crystalline product were obtained. The filtrate and washings were evaporated and the residue was dissolved in 0.5 $\text{N H}_2\text{SO}_4$ and treated with cuprous oxide as above. After decomposition of the cuprous salt another crop of the crystals, amounting to 0.9 gm., was obtained. The total yield of 2.2 gm. represented 27 per cent of the theoretical yield based on the amount of saponified condensation product used in the reduction.

The crystalline product melted at 190° and, when mixed with an authentic sample of glutathione isolated from natural sources, no depression of the melting point resulted. The specific rotation of the synthetic material was identical with that of the naturally occurring material, namely $[\alpha]_D^{27} = -21.3^\circ$ for a 2 per cent solution in water. The crystalline form of the two products was also identical. The synthetic material yielded on analysis 13.54 per cent nitrogen and 10.66 per cent sulfur. These figures are in close agreement with the theoretical values of 13.68 per cent nitrogen and 10.44 per cent sulfur for glutathione.

SUMMARY

A synthesis of glutathione has been presented. S-Benzylcysteinylglycine methyl ester was condensed with the acid chloride of α -methyl-N-carbobenzoxyglutamate. After saponification of the condensation product, the N-carbobenzoxy- γ -glutamyl-S-benzylcysteinylglycine so obtained was reduced with sodium in liquid ammonia. The resulting glutathione was isolated through the mercury and copper salts and finally as the free crystalline tripeptide.

The yields obtained in the various steps and the reagents and procedures involved in the reactions are of such a nature that the synthesis should prove to be a feasible preparative method for obtaining this physiologically important compound.

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STUDIES ON ENZYME ACTION

L. THE ESTIMATION OF PEPSIN AND TRYPSIN IN YEAST

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The chemistry of the proteolytic enzymes in yeast has been the subject of a considerable number of investigations. Among the more recent studies in which the pH values of the reaction mixtures were carefully controlled may be mentioned those of Dernby (1), of Macrae (2), and of Willstätter and Grassmann (3). Dernby showed the presence of proteolytic enzymes whose optimum pH values were 5, 7, and 7.8. Macrae showed the presence of peptidases acting at pH 5 and 7, while Willstätter demonstrated a protease acting at an optimum pH of 5. The methods used were various modifications of Sørensen's formol titration method. None of these investigators reported evidence of the presence of a pepsin or peptidase enzyme with an optimum pH in the neighborhood of 2, such as the animal pepsins described by different workers and the crystalline pepsin isolated by Northrop (4, 5). In fact, to judge from the experimental data reported, the yeast protease acting on the acid side showed practically no activity at pH 3.0 and

In this paper results will be presented showing the presence in yeast of a pepsin-like enzyme acting best at pH 1.8, and of a trypsin-like enzyme acting at pH 7.8. The various problems involved in the estimation of the protease actions and in the preparation of the yeast enzyme material will be presented.¹

EXPERIMENTAL

In view of the studies of Northrop and his coworkers of the methods of estimating protease actions, it was decided to use the

¹This yeast was very kindly supplied by Standard Brands, Inc., to whom we wish to express our thanks.

determination of the amount of tyrosine produced by the action of the enzyme material on hemoglobin as a measure of the amount of protein hydrolysis. This method is applicable to both pepsin and trypsin preparations. The conditions for the tests have been studied carefully by Anson and Mirsky (6). They defined 1 unit of pepsin or trypsin action as the formation per minute, in 6 ml. of digestion mixture, at 35.5°, of the same color as 1 milli-equivalent of tyrosine would produce. Trypsin, however, is more conveniently tested at 25° and a factor is given for the conversion of this value to 35.5°.

The Anson and Mirsky procedure for pepsin is as follows: 5 ml. of a 2 per cent hemoglobin solution and 1 ml. of enzyme solution are incubated at 35.5°, and at the end of 5 minutes 10 ml. of 4 per cent trichloroacetic acid are added and the whole mixture filtered through fine paper. To 3 ml. of this filtrate are added 20 ml. of water, 1 ml. of 3.85 N NaOH, and 1 ml. of Folin and Ciocalteau's phenol reagent (phosphotungstic acid). The standard is made up from 3 ml. of a tyrosine solution containing 0.05 mg. per ml. After 5 to 10 minutes the blue colors are read against the standard which is set at 20 mm. on the colorimeter, and the reading for the unknown is called x . For the calculations, the equation given by Anson and Mirsky,

$$\left(\frac{20}{x} (0.15 + 0.015) - 0.025 \right) \frac{16}{3}$$

times the number of times the enzyme solution was diluted gives the number of mg. of tyrosine produced by 1 ml. of enzyme solution.

For trypsin, the Anson and Mirsky procedure is as follows: 5 ml. of a denatured hemoglobin solution and 1 ml. of enzyme solution are incubated at 25° and after 5 minutes 10 ml. of 5 per cent trichloroacetic acid are added. This mixture is allowed to stand for 5 minutes and is then filtered through fine paper. To 5 ml. of this filtrate are added 10 ml. of 0.5 N NaOH, and 3 ml. of Folin and Ciocalteau's phenol reagent, diluted 1 part of reagent to 2 parts of water. The standard is made up from 5 ml. of a tyrosine solution containing 0.03 mg. of tyrosine per ml. After 5 minutes the blue colors are read against the standard, which is set at 20 mm.

on the colorimeter and the reading for the unknown is called x . In determining the tryptic action the following equation is used:

$$\left(\frac{20}{x} (0.15)\right) \frac{16}{5}$$

times the number of times the enzyme solution has been diluted. This gives the number of mg. of tyrosine produced by 1 ml. of original enzyme solution.

In applying this method to the estimation of pepsin in yeast several modifications were found to be necessary. With the Anson and Mirsky method on suspensions of yeast in water, the addition of phenol reagent to the digestion mixture filtrate

TABLE I
Effect of Increase in Alkali on Amount of Tyrosine Determined

Pepsin solution concentration, 1 ml. used	Anson and Mirsky method. Tyrosine in 16 ml. filtrate, 1 ml. NaOH used	Anson and Mirsky modification. Tyrosine in 16 ml. filtrate, 1.5 ml. NaOH used
<i>per cent</i>	<i>mg.</i>	<i>mg.</i>
0.250	2.018	2.125
0.166	2.051	2.124
0.125	2.017	2.256
0.100	2.098	2.163
Average.....	2.046	2.142

for the determination of tyrosine gave green colors instead of blue. The pH values of the yeast filtrates were tested (glass electrode) and gave wide variations from the pH values of Fairchild's pepsin filtrates and from those of the standards. The addition of 1 ml. of 3.85 N NaOH tended partially to wipe out these pH differences, while the increase in alkali from 1 ml. to 1.5 ml. of 3.85 N NaOH in both standard and yeast filtrates completely obliterated all differences and also gave blue colors.

In order to test the validity of the method with the increase in alkali, experiments were run with Fairchild's pepsin and the tyrosine in the filtrates determined with both 1 ml. and with 1.5 ml. of 3.85 N NaOH. Each of the figures in Table I is the average of several experiments.

A stock solution of 0.5 per cent Fairchild's pepsin 1:3000 was

used, and all dilutions were made from this. For the figures in the third column of Table I, the equation

$$\left(\frac{20}{x} (0.15 + 0.015) - 0.034 \right) \frac{16}{3}$$

times the dilution of the enzyme was used. Anson and Mirsky found that 0.025 mg. of tyrosine had to be subtracted in order to get true proportionality; when 1.5 ml. of 3.85 N NaOH were used, for 3 ml. of filtrate, 0.034 mg. had to be subtracted. This figure was the result of experiments with known standard tyrosine solutions whose color was determined with 1.5 ml. of 3.85 N NaOH. It represents the extraneous color-producing substances in the trichloroacetic acid filtrate even when no enzyme is present.

On application of this modified procedure to aqueous suspensions of yeast it was found that the yeast suspensions gave a constant amount of tyrosine which did not increase on incubation with denatured hemoglobin solution. Since this value did not increase and since peptic action can be represented only by an increase in tyrosine, this yeast suspension showed a blank value (tyrosine control) but no peptic action. Specifically, we have defined the blank as that value of tyrosine expressed in mg. that is present in yeast at 0 digestion time; and the peptic action is that increase in tyrosine produced in a given digestion time. In order to convert such an increase into peptic units it is multiplied by $(1/t)(1/181)$ where t is the digestion time and 181 is the molecular weight of tyrosine (4).

Since no peptic action was found in aqueous suspensions of yeast, and since it was possible that pepsin might be an intracellular enzyme, various methods of autolysis were attempted. Ethyl acetate, toluene, toluene and water, chloroform, and ether were tested. Ether gave the most satisfactory results. The other reagents showed values of peptic action ranging from zero to two-thirds of those obtained with ether. In no case could broken cells be seen upon microscopic examination after the autolysis. When 50 ml. of ether were added to 250 gm. of Fleischmann's seed yeast, the yeast liquefied within half an hour. The peptic action in this autolysis mixture increased slightly up to 5 hours of autolysis. After 5 hours it rapidly approached a maximum and then remained constant from 14 to 24 hours. The

blank value of the autolysis mixture approached a maximum in 4 hours and remained constant thereafter.

The method for preparing an enzyme solution from yeast was as follows: To 250 gm. of yeast are added 50 ml. of ether and the mixture is allowed to stand at room temperature for about 18 hours. This mixture is then centrifuged in a Sharples centrifuge (batch bowl) for 10 minutes, and the heavier liquid phase used. From 250 gm. of yeast about 90 ml. of liquid were obtained. This yeast liquor contained all of the peptic action that could be demonstrated in the yeast. The yeast liquor retained its activity for 2 hours when kept in ice water. For yeast liquor the time of digestion used was 30 minutes (Anson and Mirsky used 5 minutes for their pepsin preparations). In determining the peptic action of this yeast liquor the following equation was used

$$\left(\frac{20}{x_1} (0.15 + 0.015) - 0.034 \right) \frac{16}{3} - \left(\frac{20}{x_2} (0.15 + 0.015) - 0.034 \right) \frac{16}{3}$$

or

$$\left(\frac{3.3}{x_1} - \frac{3.3}{x_2} \right) \frac{16}{3}$$

where x_1 is the colorimeter reading of the yeast liquor filtrate after digestion and x_2 is the reading before digestion.

In carrying out the estimations it was found that the tyrosine values of the yeast autolysates (blank values) at room temperature and higher, on dilution with water, were not proportional to the concentration of the autolysate. Table II contains some values showing this lack of proportionality. The values in parentheses were calculated from the first value in each column on the assumption of proportionality. It is evident that the method of analysis showed larger amounts of tyrosine on dilution than would be expected. No reason for these differences was evident. It is, therefore, essential in carrying out protease estimations in yeast by this method to use the blank or 0 time value for the concentration of yeast liquor used in the enzyme actions.

Different diluents, such as pH 4.65 phosphate buffer, 10 per cent $(\text{NH}_4)_2\text{SO}_4$, Ringer's solution, and 0.9 per cent NaCl did not eliminate this lack of proportionality.

However, if yeast liquor was cooled to 2° immediately after

centrifugation and diluted with ice-cold water and then used at once, no increase in blank values was found, so long as the dilution did not exceed 1:3.

In Table III are presented several of the peptic actions of a number of yeast liquors from one sample of Fleischmann's seed yeast by this method. In each experiment the substrate consisted of 5 ml. of 5 per cent hemoglobin in 0.06 N HCl (6). The pH of the mixtures was 1.8.

Attempts to reduce the tyrosine blank, which usually amounted to 50 to 60 per cent of the total final tyrosine, met with little success. Dialysis was tried and in every case the peptic action

TABLE II

Tyrosine Contents of Yeast Liquors at Different Dilutions (Blank Values)

Composition		Tyrosine found		
Yeast liquor	Water	Sample I	Sample II	Sample III
ml.	ml.	mg.	mg.	mg.
1.0	0		2.704	
0.50	0.50	1.760	1.400 (1.352)	1.323
0.33	0.67			0.937 (0.882)
0.25	0.75	0.912 (0.880)	0.715 (0.676)	0.816 (0.662)
0.20	0.80			0.720 (0.520)
0.17	0.83	0.715 (0.587)	0.531 (0.451)	

The values in parentheses were calculated from the first value in each column on the assumption of proportionality.

was decreased, but with the addition of 1 ml. of 3.85 N NaOH the filtrates of these yeast liquors gave blue colors instead of green. When dialysis took place against 0.1 N HCl or H₂SO₄, the blank tyrosine value was decreased by half and the activity was totally lost. When dialysis took place against water, the blank was only slightly decreased but the activity was greatly decreased. Even so short a dialysis period as 3 hours against acid caused complete loss of peptic action. The addition of the outside water to the dialysate in the collodion bag did not restore the yeast liquor's peptic action.

A number of similar tests were carried out at pH 2.5, 3.5, and 4.6. In none of these was any appreciable action on hemoglobin found. A few experiments with edestin as substrate gave a

small amount of hydrolysis at pH 2.5, but none at pH 4.0. At pH 1.8 edestin was precipitated from the solution.

Based on these results, it may be stated that pepsin is present in crude yeast liquor in a concentration equal to 0.01 mg. per ml. of a solution of Northrop's crystallized pepsin (4).

TABLE III

Peptic Actions in Yeast Liquors at Various Dilutions

A = total amount of tyrosine present in 16 ml. of filtrate after a digestion time of 30 minutes; *B* = tyrosine present at 0 digestion time; *A* - *B* = tyrosine produced by the action of the yeast liquor on hemoglobin solution at pH 1.8 (glass electrode); peptic units are calculated back to 1 ml. of original yeast liquor $\times 10^4$.

Sample No.		1 ml. yeast liquor, 5 ml. Hb solution	0.5 ml. yeast liquor, 0.5 ml. H ₂ O, 5 ml. Hb solution	0.33 ml. yeast liquor, 0.67 ml. H ₂ O, 5 ml. Hb solution
I	<i>A</i>	6.400	2.816	1.808
	<i>B</i>	3.120	1.544	1.056
	<i>A</i> - <i>B</i>	3.280	1.272	0.752
	Peptic units	6.04	6.49	4.16
II	<i>A</i>	6.048	2.832	1.968
	<i>B</i>	3.360	1.648	1.163
	<i>A</i> - <i>B</i>	2.688	1.184	0.805
	Peptic units	4.95	4.36	3.53
III	<i>A</i>	5.280	2.496	1.576
	<i>B</i>	2.640	1.320	0.960
	<i>A</i> - <i>B</i>	2.640	1.176	0.616
	Peptic units	4.86	3.44	3.40
IV	<i>A</i>	4.736	2.055	1.341
	<i>B</i>	2.656	1.440	0.936
	<i>A</i> - <i>B</i>	2.080	0.615	0.405
	Peptic units	3.74	2.66	2.23

Samples III and IV are the same yeast, tested on the same day; Sample III was autolyzed at room temperature and Sample IV at 5°. It can be seen that there was no difference in the blank value of tyrosine, while the peptic action of the yeast liquor was decreased when the yeast was autolyzed at the lower temperature.

For the estimation of trypsin in yeast the same autolyzed yeast liquor was used. No modification of the Anson and Mirsky method was necessary. The yeast liquor contains all the tryptic action that can be demonstrated in the yeast. For the calcula-

TABLE IV

Tryptic Actions in Yeast Liquor at Various Dilutions

A = total amount of tyrosine produced by 1 ml. of yeast liquor at various dilutions in 30 minutes digestion time on a denatured hemoglobin solution at pH 7.8; * B = initial amount of tyrosine present in 1 ml. of yeast liquor at 0 digestion time; $A - B$ = amount of tyrosine produced by 1 ml. of yeast liquor in 30 minutes digestion time; tryptic units are calculated back to 1 ml. of yeast liquor $\times 10^4$.

Sample No		1 ml. yeast liquor, 5 ml. Hb solution	0.5 ml. yeast liquor, 0.5 ml. H ₂ O, 5 ml. Hb solution	0.33 ml. yeast liquor, 0.67 ml. H ₂ O, 5 ml. Hb solution
I	A	2.840	1.510	1.081
	B	2.543	1.290	0.909
	$A - B$	0.297	0.220	0.172
	Tryptic units	5.5	8.1	9.5
II	A	2.891	1.463	1.060
	B	2.547	1.311	0.899
	$A - B$	0.344	0.152	0.161
	Tryptic units	6.3	5.6	8.9
III	A	2.776	1.454	1.060
	B	2.514	1.304	0.884
	$A - B$	0.262	0.150	0.176
	Tryptic units	4.8	5.5	9.7
IV	A	2.768	1.490	1.026
	B	2.450	1.270	0.860
	$A - B$	0.318	0.220	0.166
	Tryptic units	5.9	8.1	9.1
V	A	2.760	1.470	1.032
	B	2.499	1.269	0.904
	$A - B$	0.261	0.201	0.128
	Tryptic units	4.8	7.4	7.1

* This solution is obtained by adding 40 gm. of urea to 100 gm. of a solution which contains 2.2 gm. of denatured hemoglobin and the equivalents of 10 ml. of 0.2 M KH_2PO_4 and 80 ml. of 0.2 M NaOH .

tion of the tryptic action in the yeast liquor the following equation was used.

$$\left(\frac{20}{x_1} (0.15)\right) \frac{16}{5} - \left(\frac{20}{x_2} (0.15)\right) \frac{16}{5} \quad \text{or} \quad \left(\frac{3.0}{x_1} - \frac{3.0}{x_2}\right) \frac{16}{5}$$

times the dilution of the yeast liquor gives mg. of tyrosine produced by 1 ml. of original yeast liquor. x_1 here is the colorimeter reading of the yeast liquor filtrate after 30 minutes digestion, and x_2 is the colorimeter reading of the yeast liquor filtrate after 0

digestion time. To convert this increase in tyrosine to tryptic units it is multiplied by $(1/t)$ ($1/181$), where t is the digestion time and 181 is the molecular weight of tyrosine. In Table IV are presented a series of results on Fleischmann's seed yeast by the above method. A series of experiments with one yeast sample and different autolysates from it is given in Table IV. The tryptic unit values varied to some extent, but these variations may well be ascribed to the experimental inaccuracies inherent in the tests. A number of additional samples of yeast were tested

TABLE V

Determination of Rate of Hydrolysis of Hemoglobin by Yeast Trypsin

All values are expressed in mg. of tyrosine per ml. of original yeast liquor.

Hb blank = 0; yeast blank = 1.987.

Time	Yeast and Hb digestion	Tyrosine produced	Protein unchanged	k_{25}^*
<i>min.</i>				
0	1.987	0	11.40	
30	2.365	0.378	11.12	0.00111
60	2.758	0.771	10.63	0.00118
90	3.037	1.050	10.35	0.00108
120	3.374	1.387	10.01	0.00109
150	3.704	1.717	9.68	0.00109
180	3.918	1.931	9.47	0.00104
210	4.371	2.384	9.01	0.00112
240	4.506	2.539	8.86	0.00105

The average value of k for this experiment is 0.00110. The protein unchanged at 0 time was determined by acid hydrolysis of 1 ml. of yeast liquor and 5 ml. of hemoglobin solution at pH 7.8.

similarly and gave analogous results. In some cases, the calculated values of tryptic units varied more, but these differences must be looked upon as accidental. The large values of the blanks and the necessity for rapid working play an important part in these accidental variations.

The results of an experiment in which the yeast trypsin was allowed to act on hemoglobin for 240 minutes and the extent of the action measured at intervals are presented in Table V. The values of the velocity constant k as determined by the equation

$$k = 1/0.4343t(\log a/(a - x))$$

where a was the total tyrosine that could be produced from all the protein present and x was the amount of tyrosine produced in any given time by the yeast liquor were calculated. This x was found by subtracting the amount of tyrosine found at 0 time from that found at t time. The blank (0 digestion time) was determined in two ways. 1 ml. of the enzyme solution and 5 ml. of 7.3 buffer were allowed to stand for t time at 25° and then precipitated with 10 ml. of 5 per cent trichloroacetic acid; or 1 ml. of yeast liquor was allowed to stand for t time at 25° and then 5 ml. of hemoglobin solution and 10 ml. of 5 per cent trichloroacetic acid were added. Both gave identical results. In carrying out

TABLE VI
Determination of k_{25} for Yeast Liquor and for Yeast Liquor Diluted

Time	1 ml. yeast liquor	0.5 ml. yeast liquor, 0.5 ml. H ₂ O
min.	k	k
30		0.00129
60	0.00136	0.00130
90		0.00126
120	0.00144	0.00122
150		0.00127
180	0.00145	0.00128
210		0.00121
240		0.00115
Average.....	0.00142	0.00124

the experiments a number of tubes were set up, each tube containing 5 ml. of 5 per cent hemoglobin solution at a pH of 7.8 and 1 ml. of yeast liquor. These were allowed to stand at 25° . At intervals (shown in Table V) the tyrosine was determined in a blank and in a yeast liquor-hemoglobin solution.

Table VI shows a series of values of k for a different yeast liquor undiluted and the same yeast liquor diluted with an equal quantity of water. It is evident that k is constant for the given conditions; in other words, the action follows the monomolecular reaction velocity law. The values of k were somewhat larger than those given in Table V, but were of the same order of magnitude.

In the yeast trypsin experiments the tests were carried out only at pH 7.8. In view of the work of others who have described the presence of a trypsin-like enzyme in yeast and studied its actions at different hydrogen ion concentrations, it was not considered necessary to duplicate their material.

DISCUSSION

The experiments reported in this paper indicate the presence in yeast of a pepsin acting best at pH 1.8. This enzyme has not been described previously. Possibly the reasons for its not being found by others were the inactivation of the yeast pepsin by the addition of water at room temperature (and higher), the difficulty of finding suitable methods of autolysis by which pepsin would not be inactivated, and, in general, the instability of the pepsin as it exists in the yeast. The results obtained in this investigation showed a considerable concentration of the pepsin, the concentration in crude yeast liquor, to judge by its action, being of the order of magnitude of one-tenth of the crystallized pepsin described by Anson and Mirsky (6).

A number of experiments on the trypsin of yeast were also given. The trypsin concentration was found to be considerably less than that of the pepsin.

The method used to estimate the protease actions was the formation of tyrosine from hemoglobin according to the method of Northrop and of Anson and Mirsky. The blank values of the yeast autolysates were also large, and no method of decreasing them without destroying the pepsin and trypsin activities was found.

SUMMARY

Evidence for the existence in yeast of a pepsin acting at pH 1.8 was presented. Methods of autolysis of yeast in order to determine the most satisfactory procedure for obtaining pepsin and trypsin preparations were described. A number of experiments on the yeast pepsin and trypsin were described.

We wish to express our gratitude to Dr. K. G. Falk for his suggestion of the problem and for his advice and encouragement. We are also indebted to Miss Grace McGuire for her guidance.

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THE RELATIONSHIP OF VITAMIN C TO GLUCOSE TOLERANCE IN THE GUINEA PIG*

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The specific types of chemical change through which vitamin C functions in living tissues are only vaguely understood at the present time. From the meager evidence available, however, it is generally accepted that the vitamin plays an important rôle in tissue respiration. Its direct and indirect relationships to specific enzyme systems have been demonstrated in a number of laboratories (1). Recently, Barron, DeMeio, and Klemperer (2) have demonstrated in particularly clear cut fashion the catalytic rôle of copper and hemochromogens in the aerobic oxidation of the vitamin, and Borsook and his associates (3) have shown the importance of glutathione as a reductant and protective agent for the reversible oxidized form, dehydroascorbic acid.

With the onset of scurvy, Mosonyi and Rigo (4) have noted a parallel decrease in the oxygen consumption of guinea pigs, which presumably represents the decrease in total respiration due specifically to a deficiency of the vitamin. In studying the influence of vitamin C level upon resistance to diphtheria toxin, King and Menten (5) observed that with severe tissue injury there was a marked decrease in glucose tolerance in guinea pigs, but only a moderate disturbance in the oxygen uptake of the tissue slices studied *in vitro*. An intimate relationship between the complex lipids and the vitamin *in vivo* has been frequently observed (6-9), but only one important paper, based upon studies *in vitro*, has appeared to link the vitamin with amino acid metabolism (10).

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The present investigation was for the purpose of finding whether the vitamin C level alone would measurably influence the capacity of guinea pigs to metabolize glucose, as demonstrated by the standard type glucose tolerance test. Such a relationship has been demonstrated for both the prescorbutic and the scorbutic stages of deficiency, followed by an observed rapid return to normal when the depleted animals were supplied with the vitamin.

TABLE I
Effect of Vitamin C Depletion and Recovery on Glucose Tolerance of Guinea Pigs

Depletion period	Mg. glucose per 100 cc. blood after				No. of animals	Average weight	Minimum and maximum weights
	Fasting	40 min.	80 min.	120 min.			
<i>days</i>						<i>gm.</i>	<i>gm.</i>
Controls (2 mg. per day)	101 \pm 0.9*	149 \pm 3.9	141 \pm 3.7	95 \pm 1.4	15	583	480-718
10	104 \pm 1.4	169 \pm 3.3	161 \pm 4.7	108 \pm 3.0	14	615	470-740
15	109 \pm 1.6	176 \pm 4.3	177 \pm 4.7	131 \pm 2.9	15	593	437-747
20	112 \pm 1.3	185 \pm 4.2	191 \pm 5.1	150 \pm 5.4	15	507	337-695
<i>Recovery period</i>							
10	101 \pm 1.5	163 \pm 4.4	144 \pm 6.0	109 \pm 3.4	13	499	327-707
15	99 \pm 0.9	156 \pm 2.1	139 \pm 1.6	100 \pm 1.4	13	528	347-720

* The probable errors were calculated as outlined in Sherman (12).

EXPERIMENTAL

Guinea pigs weighing about 300 gm. were fed the vitamin C-free basal diet of Sherman and collaborators (11), supplemented with cod liver oil and a solution of crystalline vitamin C (2.0 mg. per day) until they had reached approximately 600 gm. in weight. The larger animals are much more satisfactory for blood sugar work than animals of the size generally used for vitamin assay, and the long period of standardization permits more uniform results. The animals were then depleted of their vitamin C reserves for 20 days, glucose tolerance tests being made on the 10th, 15th, and 20th days of depletion (Table I). At the end of

20 days depletion the animals were fed 10 mg. of vitamin C per day to test their capacity for regaining a normal response to the glucose tolerance tests. It will be noted that on the 10th day recovery was fairly satisfactory, and that by the 15th day the response was practically normal.

Blood sugar was determined by the Folin-Malmros micro-method (13). Samples were obtained from a marginal ear vein after piercing with a Bard-Parker No. 11 pointed blade. The animals were fasted for 2.5 hours preliminary to the glucose tolerance tests. The fasting blood sugar samples were then taken and the animals were fed 1.75 gm. of glucose per kilo of body weight (40 per cent glucose solution). Additional blood samples were taken at 40, 80, and 120 minute intervals after feeding glucose. Fasting times up to 6 hours did not cause a marked change in the normal glucose tolerance curve. Comparisons against standard glucose solutions were made with a Duboscq colorimeter with a yellow light filter.

DISCUSSION

From the differences and probable error values given in Table I, it is evident that there is a high probability that the differences noted are real. For example, after 10, 15, and 20 days depletion, the calculated probability that the successive increases at the 120 minute period were real, would be 140:1, 2000:1, and 25:1 respectively. At the end of 10 days depletion the animals were still gaining weight and showed no external evidence of being abnormal. The highest weight levels were reached between the 10th and 15th days of depletion, followed by a rapid loss in weight, between the 15th and 20th days of depletion, and the onset of scurvy.

The normal glucose tolerance curve for guinea pigs reaches a peak at approximately 40 minutes after feeding. As the animals become depleted of vitamin C the blood sugar value at 40 minutes is considerably higher than that of the normal, and the peak shifts to the 80 minute period. There is a progressive and characteristic shifting of the curve upward and to the right concurrently with depletion. After 15 days of vitamin readministration, the position of the curve was practically normal. It is evident from other investigations in our laboratory that compar-

able results are not obtained in dealing with deficiencies of some of the other vitamins (thermolabile factors in the vitamin B group). A specific explanation of the observed phenomena, correlating the vitamin C level of nutrition with glucose tolerance, is not apparent at the present time, but it appears significant that vitamin C is normally present in high concentration in the tissues which control glucose utilization (*e.g.* pituitary, pancreas, adrenal, thyroid, liver, intestinal wall). A deficiency may thus cause a suppression in general respiratory processes, an alteration in capillary permeability, and a decreased hormone secretion, simultaneously.

SUMMARY

Successive stages of vitamin C depletion (10, 15, and 20 days) induce a corresponding rise in fasting blood sugar level and a distinctly lowered glucose tolerance. The typical peak in the blood sugar curve moves characteristically upward and to the right with successive stages of vitamin C deficiency. Readministration of 10 mg. of vitamin per day, after 20 days depletion, induces a return to normal within 15 days.

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THE STARCH ISOLATED FROM PLANT MATERIAL BY THE FREEZING METHOD

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It has long been realized that, besides being one of the most common forms in which the plant stores food material, starch also serves as a transitory reserve material from which may arise a variety of substances, some of which are of importance for the building of the structural elements of the plant. The similarity in chemical composition of many of the substances in question makes it difficult to isolate them in pure form. This is essential, however, in order to gain reliable information concerning their constitution. We (1) reported on a procedure for isolating starch from leaves by means of a freezing method which yielded products of a higher purity than were obtained by other means and, because of its simplicity, offered various advantages. The starches which have been obtained by means of this method from leaves and from other plant material have now been examined more critically with a view to determining the purity of the products and the limitations of the method. Precipitation of starch by means of ethanol or other organic solvents from water extracts of plant material results in starch preparations which are contaminated with substances of similar chemical and physical properties. It was important to determine whether or to what extent the starch prepared by the freezing method was similarly affected.

Pectin, Gum Arabic, and Glucose—A 0.5 per cent solution of gum arabic was subjected to the same procedure followed for the separation of starch from solution by freezing at -8° for 4 days (1). However, on thawing none of the gum had separated out. Although the original solution gave only a very slight gray coloration with iodine, after the thawed solution was centrifuged, a very small amount of blue-staining starch and a perfectly clear

solution which gave no color with iodine were obtained, indicating the presence of a minute amount of starch in the gum arabic used. A commercial preparation of pectin gave similar results, a small amount of starch or dextrin being recovered from the thawed solution.

The pectin was purified by dissolving in water and precipitating with ethanol. The separated solid pectin was twice redissolved and reprecipitated with ethanol. Solutions containing 0.5 per cent of this pectin preparation and 0.5 per cent of potato starch in one case, and 0.5 per cent of gum arabic with 0.5 per cent of potato starch in another were frozen 4 days at -8° . The starch was then isolated from the thawed solutions in the manner pre-

TABLE I

Analysis of Gum Arabic, Pectin, and of the Starches Separated from Mixtures of These by Freezing

Substances	Ash	Nitrogen	CO ₂	Relative reducing power
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Original starch.....	0.252	0.00	0.29	100.00
Gum arabic.....	2.738	0.21	2.97	64.29
Starch separated from gum arabic.....	0.286		0.31	98.61
Pectin.....	6.992	0.09	12.73	33.06
Starch separated from pectin.....	0.452		0.41	98.52

viously described (1). The starch recovered from the pectin solution amounted to 98.8 per cent and from the gum arabic solution to 99.2 per cent. The filtrates from these solutions still contained small amounts of starch, as indicated by the iodine test. After a further period of freezing of 33 days in the case of the pectin solution and of 19 days in the case of the gum arabic solution, the remainder of the starch separated quantitatively. The analyses (Table I) indicate the purity of the starch isolated from these mixtures; the CO₂ determinations were made by means of the method of Lefèvre and Tollens (2, 3). As heretofore, the relative reducing power represents the reducing power of the starch solution, hydrolyzed for 5 hours at 100° with 1 per cent hydrochloric acid, expressed in per cent of the reducing power of the original potato starch solution.

A solution containing 0.5 per cent of potato starch and 5 per cent of glucose was frozen for 4 days at -8° . The starch which remained on thawing was washed with water five times by centrifugation and was dried. A 1 per cent solution of this starch gave no trace of reduction with Benedict's solution, indicating that the starch had been completely separated from the glucose by freezing.

Dextrins—A very much more difficult situation is presented when dextrins are associated with starch. The number and nature of these intermediate products of the hydrolysis of starch have not been established, and no satisfactory criteria are known for their identification or separation, although they may appear wherever starch exists in the plant. Of special importance for the present purpose is the fact that the different members of this group of carbohydrates show widely different solubility in water; some are sparingly soluble, others easily soluble, some are soluble only at higher temperatures, others at ordinary temperatures (4, 5). It is, therefore, impossible to effect a separation of these substances from starch by means of their solubility in water. With iodine the various dextrins produce substances ranging in color from blue, violet, red, orange to colorless. Doubtless in most of the methods which have been proposed for the quantitative determination of starch in plant material the results include varying amounts of dextrin. In general, the dextrins appear to be rather more reactive than starch; they reduce cupro-alkaline solutions in varying degrees. Our experiments with various dextrin preparations have shown that some of these are separated from solution by freezing to the extent of as much as 65 per cent, while others remain completely in solution. It was obvious, therefore, that the dextrins may constitute a contaminant in the preparations of starch obtained by the freezing method, and it seemed important to determine by analysis whether and to what extent this was the case.

The principle of the method which was finally evolved for the quantitative determination of starch in the presence of dextrin was suggested by the method proposed by Small (6) and depends upon the difference in solubility of the iodides of starch and dextrin in a solution of calcium chloride. After considerable preliminary experimentation calcium chloride was decided upon

as the best flocculating agent. Calcium chloride was preferred to ammonium sulfate, used by Small, because the latter salt interferes in the determination of the reducing sugars by means of Benedict's solution, and the method of determining starch by means of the reducing power of its products of acid hydrolysis proved to be more satisfactory than the one based upon polarimetric measurements as used by Small.

It is necessary to have clear solutions, and this was attained by preparing the starch mixtures at 120°. It is also essential that the solutions should be free from any other substances which precipitate in the presence of calcium chloride and the potassium iodide-iodine reagent, and which, upon hydrolysis with 1 per cent hydrochloric acid, yield products which reduce Benedict's solution. The solutions to be analyzed should contain between 25 and 60 mg. of starch in a volume of 20 cc. If the starch content is greater, a dilution should be made; if it is lower, a correspondingly greater volume of solution should be taken, the starch iodide precipitate being accumulated stepwise if necessary.

To the sample to be analyzed is added an excess of a water solution containing 4 per cent of iodine and 6 per cent of potassium iodide. To this is added 1 cc. of a 40 per cent calcium chloride solution for each 20 cc. volume. This flocculates the iodide precipitate. The precipitation is carried out in 50 cc. centrifuge tubes. The contents are centrifuged and the supernatant liquid is poured off through a small filter. The precipitate is resuspended in 20 cc. of water, and 1 cc. of the 40 per cent calcium chloride solution is added with stirring. The centrifuging and washing operations are repeated until the filtered washings no longer show a pronounced blue or red color upon the addition of more iodine solution. Five washings suffice to remove the excess iodine and potassium iodide from the precipitate. The number of further washings required depends upon the nature and quantity of dextrans present in the sample. Synthetic mixtures of starch and Merck's Reagent dextrin required ten washings to remove the dextrin when 10 per cent was present, fifteen washings for 30 per cent, and twenty washings for 50 per cent dextrin. Some naturally occurring mixtures required twenty-five to thirty washings to remove all the dextrin iodide. All washings are filtered through the same filter; any small particles

of precipitate which escape settling in the centrifuging are thereby collected. The precipitate on the filter is dissolved with boiling water and washed into the centrifuge tube which contains the bulk of the precipitate. This is suspended in water and the solution is boiled until all iodine is expelled, which usually requires 30 to 40 seconds. The iodine can also be removed by extraction with ethanol; the same results are obtained by this means, though more time is consumed. Allowing the separated precipitate of starch iodide to stand for some time, *e.g.* overnight, has little effect if the temperature is low, as in a refrigerator. However, the starch iodide should be in solution as short a time as possible, as under these conditions changes occur which lead to low results.

The starch solution, freed of iodine, is transferred quantitatively to a 25 cc. volumetric flask and hydrolyzed with 1 per cent hydrochloric acid. The time required, at 100°, to attain the maximum reducing power of the solution has been found to be 3 hours for ordinary starches and 4.5 to 6 hours for retrograded starches. The iodine procedure does not affect the time required for complete hydrolysis. From the reducing power of this solution the starch content of the original sample can be calculated. Dextrin can be determined only by difference from the reducing power of the hydrolyzed original solution. The presence therein of substances other than dextrin and starch, which yield reducing products upon hydrolysis, will, of course, constitute a source of error.

It is realized, of course, that because of our limited knowledge of the chemistry of starch and of the products formed in the early stages of its hydrolysis, the differentiation on the basis of the foregoing method may be arbitrary or imperfect. The method itself defines the nature of the substance which is here considered to be starch. Whether under this concept of starch are included some of the very first products of hydrolysis it is impossible to say. The method does, we believe, separate starch from a mixture of substances of variable behavior which are commonly classed as dextrans and which may be the source of serious error in the determination of starch.

Synthetic mixtures of potato starch with 0, 10, 30, and 50 per cent Merck's Reagent dextrin yielded results of 99.66 per cent

of starch recovered by this method. In the series of trials with known mixtures of starch and dextrin the values for starch recovered through analysis ranged from 97.19 to 99.66 per cent. Experience with the method indicates that it is accurate to within 3 per cent.

That the starch in the starch iodide is apparently partially hydrolyzed when the latter is heated is shown by the following. Starch iodide was prepared from 3 gm. of starch. One-half of the preparation was freed of iodine by boiling in water solution (Preparation A); the other half was freed from iodine by extraction with ethanol (Preparation B). The recovered, iodine-free starches were analyzed by the calcium chloride-iodine method and CO_2 determinations were made. Preparation A yielded 94.66 per cent starch by analysis and 0.13 per cent CO_2 , and Preparation B 98.35 per cent starch by analysis and 0.09 per cent CO_2 .

Leaf Starch-Dextrin—The same leaf starches, hereinafter called starch-dextrins, which had previously been isolated by the freezing method (1) were analyzed by the method just described in order to determine the amount of dextrin they contained. (The starch from *Malva parviflora* is not included as there was not a sufficient amount available.) The analyses disclose the fact that in some cases the material isolated from some of the leaf material consisted in a large part of starch, while in other cases it consisted entirely of dextrin. The analyses are given in Table II; in Column 2 are given the values for the material (starch-dextrin) frozen out of the hot water extract of the plant material in per cent of the weight of the dry leaf material after extraction with ethanol and petroleum ether; in Column 3 are given the reducing values of Benedict's solution after hydrolysis with 1 per cent hydrochloric acid for 5 hours, expressed in per cent of the reducing power of purified, retrograded potato starch; Column 4 gives the per cent of starch in the starch-dextrin isolated by freezing. The results show that on the basis of the method here used for separating starch from dextrin both starch and dextrin are precipitated by the freezing method from hot water extracts of leaves.

In order to show the values obtained by different methods of the starch content of leaves, some results on sunflower leaves are here included. The leaves were taken in the afternoon from

mature plants growing in the garden, as nearly as possible selected for a uniform sample, and divided into two lots of about 600 gm. each. The leaves in each lot were cut in half along the midrib, one-half of each leaf was immediately placed in methanol, and the other half was subjected to the vapors of chloroform in a closed container for 20 minutes. The leaves in methanol were extracted of their pigments with hot methanol and petroleum ether in the usual manner, dried, ground to pass a 60 mesh screen, and dried in a vacuum over phosphorus pentoxide. The leaves treated with chloroform were dried in an air stream at 45-50°, ground, extracted with methanol and with petroleum ether, and

TABLE II

Composition of Starch-Dextrin Obtained from Various Leaf Sources by Freezing Method

Leaf source (1)	Material frozen out (2)	Relative reducing power (3)	Starch content by iodide method (4)
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Potato (tuber).....		100.00	99.66
<i>Nicotiana tabacum</i>	37.73	98.94	93.87
<i>Phaseolus coccineus</i>	13.39	90.90	None
<i>Linum usitatissimum</i>	2.26	97.75	"
<i>Hedera helix</i> (old).....	2.23	96.54	87.90
" " (young).....	1.49	96.28	91.05
<i>Stellaria media</i>	2.23	100.27	90.44
<i>Echinocystis fabacea</i>	2.65	99.67	None

treated as the previous lot. About 1 gm. samples of the dried leaf material were extracted seven times with water at 20°. These extracts gave a light blue and later a red-brown color with iodine but they were found to contain no starch when analyzed by the calcium chloride-iodine method. The material was then extracted with 20 cc. of water at 120° for 30 minutes, followed by four additional extractions at 100°. The combined extracts were analyzed by direct hydrolysis with 1 per cent hydrochloric acid and determination of the reducing power. These values represent the total for starch and dextrin. The same extracts were also analyzed by the calcium chloride-iodine method for starch alone. Samples of 25 gm. were taken for the extraction of starch from

the leaf material by the freezing method. The results of the analyses are given in Table III and show that the values obtained from the reducing power of the hydrolyzed hot water extract are higher and more variable than those obtained by the calcium chloride-iodine method. It would also appear that a considerable amount of dextrin was included in the starch-dextrin isolated by the freezing method.

Bean, Banana, Sweet Potato, and Locust Wood—In order to test out further the applicability of the freezing method for the isolation of starch, a number of plant tissues were used which were of widely different nature and presented various difficulties for analytical purposes. The navy beans, seeds of *Phaseolus vulgaris*,

TABLE III
Starch-Dextrin and Starch Content of Sunflower Leaves in Per Cent of Dry Weight of Leaf Material Extracted with Methanol and Petroleum Ether

Lot No.	Killed with	Starch-dextrin by reducing power of hydro- lysed hot water extract	Starch by calcium chloride- iodine method	Starch-dextrin isolated by freezing
		per cent	per cent	per cent
1	CHCl ₃	6.91	4.65	5.84
1	CH ₃ OH	7.43	4.29	5.93
2	CHCl ₃	6.68	4.58	5.82
2	CH ₃ OH	7.81	4.56	6.29

were ground finely, so that the material passed through a 100 mesh screen; they were thoroughly extracted alternatively with ethanol and petroleum ether and dried in a vacuum over calcium chloride. Sweet potato, *Ipomœa batatas*, was sliced and partially dehydrated with ethanol. The slices were ground, dehydrated further with ethanol, and ground to pass a 100 mesh screen, extracted with petroleum ether and ethanol, and dried in a vacuum over calcium chloride. Very green bananas, the fruit of *Musa sapientum*, were peeled, sliced thin, and treated in the same manner as the sweet potato. The roots of black locust, *Robinia pseudoacacia*, collected in October by Dr. I. W. Bailey, at Norwell, Massachusetts, were completely freed of bark, and pieces about 1 cm. in diameter were rasped to a powder, dried, ground in a

pebble mill to pass a 100 mesh screen, extracted, and dried in the same way as the other material. In Table IV are given the analyses of these plant materials, the starch-dextrin representing values for this as obtained by the freezing method and the last column being the values obtained for starch in the hot water extracts by means of the iodine-calcium chloride method. The

TABLE IV

Analysis of Various Plant Materials: Starch-Dextrin Obtained by Freezing Method and Starch Content by Iodine-Calcium Chloride Method

Material	Ash	Nitrogen	CO ₂	Starch-dextrin isolated	Starch content
	per cent	per cent	per cent	per cent	per cent
Beans.....	5.83	3.67	0.90	37.68	34.94
Bananas.....	2.40	1.03	0.70	70.80	66.31
Sweet potato.....	3.90	0.79	1.22	60.80	54.56
Locust wood.....	5.35	0.78	0.80		
Cold water.....				14.36	0.00
Hot ".....				3.66	5.46

TABLE V

Analysis of Starch-Dextrin Obtained from Various Plant Materials by Freezing Method

Material	Ash	Nitrogen	CO ₂	Starch in starch-dextrin
	per cent	per cent	per cent	per cent
Beans.....	0.22	0.27	0.35	86.23
Bananas.....	0.06	0.17	0.21	94.20
Sweet potato.....	0.12	0.05	0.35	94.28
Locust wood				
Cold water.....	0.52	0.20	0.32	0.00
Hot ".....	1.70	0.15	0.29	84.74

values show to what extent the starch contained in these plant materials can be isolated by means of the freezing method. In Table V are given the results of the analyses of the starch-dextrin which was isolated. They show that varying amounts of starch are contained in the starch-dextrin isolated by freezing. The most striking is the case of the cold water extract of black locust wood which on freezing yielded a considerable quantity, 14.36

per cent of the wood extracted, consisting of pure white, blue-staining material, which, however, on the basis of the iodine-calcium chloride method proved to contain no starch.

It should be emphasized that some plant material presents difficulties in the complete extraction of the starch by means of water at 100° and even at 120°. In such cases, of which the bean is an example, no starch is removed after the first two or three extractions, although the residual material may stain an intense blue-black with iodine. From such material the starch can be extracted by means of a solution of calcium chloride as described by Denny (7) and can be precipitated and freed of calcium chloride with ethanol.

SUMMARY

Starch can be separated by the freezing method from pectin, gum arabic, and glucose without serious contamination. Some of the dextrans, however, are also precipitated on freezing, and in hot water extracts of plant material may constitute a large proportion of the material separated by the freezing method. The amount of dextrin accompanying the starch separated from plant extracts by the freezing method has been estimated by means of an analytical procedure based upon the differential solubility of the iodides of starch and dextrin in a solution of calcium chloride. The starch-dextrin mixtures separated from a number of plant sources have been examined by this means.

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THE PHOSPHOLIPID FATTY ACIDS OF MUSCLE

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In his studies of the lipids of beef tissue, Bloor (1, 2) noted a correlation between the physiological activity of a tissue and its phospholipid content, the more active tissue having the larger percentage of phospholipid. A similar correlation (3) was later found to exist between the unsaturation of the fatty acids of the organs and their physiological activity, a relation, at that time, not found for the muscles. In the tissues other than muscle, the percentage of arachidonic acid and the iodine number of the liquid fatty acids varied inversely as the percentage of oleic acid (Table I).

Other workers (4-7) have confirmed the observation that physiological activity and phospholipid content are related, but no other known studies have been made concerning the relation of activity to the unsaturation of the fatty acids.

As the result of a study of the fatty acid distribution in rats on various diets, Sinclair (8-10) found that while the iodine numbers of the phospholipid fatty acids of the liver could be raised very rapidly by the feeding of a highly unsaturated fat, the change in the phospholipid fatty acids of skeletal muscle was comparatively slow. He concluded that the liver phospholipid was of two types, metabolic and structural, while that of the muscles was essentially structural. He further observed that the ratio of liquid to solid fatty acids was constant regardless of the degree of unsaturation of the liquid acids.

Recent studies (11, 12) carried on in this laboratory showed definite differences in the phospholipid and cholesterol content of the same muscles of the wild and laboratory rabbit and of the fowl and pigeon, differences apparently due to variations in muscular

activity in the animals under different natural conditions. In the present problem, an attempt was made to find out whether the ratio of liquid to solid fatty acids and their degree of unsaturation in the phospholipid of muscles, submitted to varying amounts of activity by exercise, changed with the activity or whether these characteristics were inherent and constant.

Muscle is especially suitable for studies of this kind for two reasons: (a) It has a single function, namely to convert fuel into mechanical work; (b) different muscles vary in their degree of activity.

TABLE I
Unsaturation and Physiological Activity in Beef Organs

Tissue	Lecithin			Cephalin		
	Oleic acid	Arachidonic acid	I No.	Oleic acid	Arachidonic acid	I No.
	<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>	
Liver.....	17	21.0	181	28	19.0	200
Kidney.....	43	12.0	173	40	11.0	180
Pancreas.....	51	11.4	140	55	9.7	154
Lung.....	58	5.0	136	60	7.0	149

Methods

The animals studied included 8 laboratory rabbits, 6 wild rabbits, 3 cats, 3 dogs, 9 guinea pigs, 14 pigeons, 3 hens, 2 roosters, and 3 owls.

Exercising the guinea pigs was accomplished by placing them in a treadmill for 6 hours a day from 4 to 6 weeks, while matched litter mates were kept in small cages for the same length of time. Young pigeons which had never flown were likewise housed in small cages from 2 months to 1 year, while others of the same age acted as controls and were allowed to fly freely.

Preparation of Sample—The animals were killed by bleeding, the muscles quickly removed, freed from visible fat, and the tissue extracted in the usual manner (13).

Phospholipid—The phospholipid determination followed the Bloor oxidative procedure (14) with the following modifications: (a) The saturated magnesium chloride solution was replaced by a

4 per cent solution; (b) the period of centrifugation was reduced from 5 to 2 minutes in the first case and from 3 minutes to 1 minute in the second; (c) the acetone was dried over calcium chloride previous to distillation to insure a better precipitation of the phospholipid; (d) the oxidized material was cooled before the addition of iced water to prevent loss of iodine by volatilization after the addition of potassium iodide.

Fatty Acids—The phospholipid was saponified according to the Bloor technique (13). The fatty acids were extracted with petroleum ether and made up to a volume of 50 cc.

Iodine Numbers—The iodine numbers were determined by the Yasuda modification (15) of the Rosenmund-Kuhnnehn pyridine sulfate dibromide method (16).

Separation of Liquid and Solid Fatty Acids—The liquid and solid fatty acids were separated by Sinclair's micro lead soap method (8), a modification of the Twitchell procedure (17).

Pitchy Residue—In the separation of the liquid and solid acids, a "pitchy residue" fraction was isolated and determined according to the Sinclair technique (8).

Results

Since the iodine numbers of the fatty acids of the individual muscles of all the animals examined were found to be very similar, only certain typical analyses have been recorded (Tables II and III). Together with these the few atypical values are given.

Mixed Fatty Acids—In Table II are given the iodine numbers of the mixed fatty acids of typical muscle from all the animals examined. The values for the wild rabbit were somewhat higher in most of the muscles than those of the laboratory rabbit, but this variation might be referred to difference in diet. The same is probably true for the differences displayed in the iodine numbers of the cat and dog.

No variation was found in the degree of unsaturation of the mixed fatty acids between the exercised and unexercised guinea pigs, which indicates that an increase in activity, at least of short duration, does not affect the nature of the phospholipid fatty acids in muscle. The iodine numbers of the phospholipid fatty acids in the flying and resting pigeons were likewise strikingly constant with but one exception—the forearm showed a definitely higher

degree of unsaturation in the fliers (Table II). What significance this high value has in view of the other figures cannot be stated.

The iodine values for the mixed fatty acids of pigeon muscle were higher on the whole than those of the hen, but the differences were not striking enough to permit the conclusion that there is a definite correlation between activity and unsaturation.

TABLE II
Iodine Numbers of Mixed Fatty Acids of Muscle Phospholipid

Animal	No. of animals	Muscle	Mixed fatty acids					
			Dry weight			I No.		
			Average	Low	High	Average	Low	High
			per cent	per cent	per cent			
Rabbit								
Wild	6	Back	2.4	1.9	3.2	132	126	143
Laboratory	8	“	1.27	0.9	2.5	116	108	129
Guinea pig								
Exercised	4	Abdominal wall	2.4	2.1	2.6	125	108	143
Unexercised	5	“	2.0	1.7	2.5	131	110	153
Pigeon								
Fliers	7	Pectoralis major	3.6	3.3	4.2	136	122	139
		Forearm	3.0	2.7	3.8	147	142	154
Non-fliers	7	Pectoralis major	3.2	2.2	4.3	134	116	144
		Forearm	2.9	2.5	3.2	128	121	136
Owl	3	Pectoralis major	3.2	3.0	3.7	123	115	132
Hen	5	“	1.3	1.1	1.5	126	119	134
Dog	5	Diaphragm	2.4	1.6	3.0	133	121	146
Cat	3	Jaw	2.4	2.3	2.6	116	111	121

Liquid and Solid Fatty Acids—Sinclair's recent modification of the Twitchell lead soap method made it possible to study the distribution of the liquid and solid fatty acids in muscle. Since this method is quite recent and since some of the muscles were too small to permit such an extensive study, the series is not as complete as that of the mixed fatty acids. Some of the results were obtained while the method was still being perfected and are therefore more variable than is desirable, but they do show the relation between the two fractions (Table III).

The fraction referred to as pitchy residue or as a solid unsaturated acid in previous publications (1, 2, 18) was likewise evident

TABLE III
Distribution of Phospholipid Fatty Acids of Muscle

Muscle		Mixed fatty acids			Liquid fatty acids			Solid fatty acids			Pitchy residue	
		Phospholipid	Moist weight	I No.	Mixed fatty acids	Moist weight	I No.	Mixed fatty acids	Moist weight	I No.	Mixed fatty acids	Moist weight
Typical values												
Pectoralis major (pigeon)	Average	63.5	1.01	136	71.0	0.69	175	27.0	0.26	5.0	4.2	0.049
	Low	60.0	0.88	120	70.0	0.63	170	24.0	0.25	0.0	3.0	0.040
	High	73.0	1.20	140	75.0	0.86	176	29.0	0.31	8.6	6.4	0.055
Back (guinea pig)	Average	64.0	0.52	134	74.0	0.39	168	26.1	0.127	4.4	6.5	0.034
	Low	60.0	0.42	114	62.0	0.23	155	15.0	0.065	0.0	3.0	0.010
	High	70.0	0.63	150	81.0	0.56	193	39.0	0.140	8.0	11.0	0.080
Neck (dog)	Average	70.0	0.66	125	73.0	0.45	165	23.0	0.10	10.0	5.3	0.030
	Low	67.0	0.57	115	69.0	0.41	154	16.7	0.14	2.0	3.9	0.022
	High	74.0	0.74	131	77.0	0.48	175	28.5	0.20	17.0	6.1	0.040
Gastrocnemius (hen)	Average	64.0	0.47	135	76.8	0.34	171	25.6	0.11	8.3	7.2	0.036
	Low	63.0	0.41	132	70.0	0.31	157	17.6	0.07	6.5	4.6	0.020
	High	66.0	0.52	141	84.0	0.39	184	29.0	0.14	10.9	9.4	0.050

Atypical values

Forearm (pigeon)*	Average	66.0	0.97	147	70.0	0.66	204	28.0	0.27	10.6	4.1	0.036
	Low	65.0	0.91	142	69.0	0.62	181	27.0	0.25	0.0	3.8	0.023
	High	70.0	0.99	154	73.0	0.69	208	29.0	0.29	12.6	6.0	0.039
Heart (hen)†	Average	68.9	1.36	125	72.3	0.91	156	21.3	0.27	22.0	7.1	0.097
	Low	66.0	1.31	113	64.0	0.87	146	19.0	0.25	15.0	4.1	0.060
	High	72.0	1.40	150	75.0	0.97	157	21.0	0.28	30.0	10.0	0.140

* Iodine number higher than average.

† Iodine number lower than average.

in the present analyses. Its composition is unknown, but it is thought to be sphingomyelin or a mixture of it with cerebrosides or their partially split products. Since the substance was not fatty

acid in nature, the amount was subtracted from the total weight of the mixed fatty acid fraction before calculating the percentage of liquid and solid fatty acids. The percentage of pitchy residue itself was calculated from the total weight of the mixed fatty acids. Iodine numbers were not determined routinely, since the nature of the substance is unknown and the values would therefore have little meaning.

Before one attempts to interpret the results of these analyses, a few facts concerning the separation of the mixed fatty acids require explanation. The method recommends the use of at least 30 mg. of mixed fatty acids for the separation into liquid and solid acids, but in several instances this amount was not available so that amounts of 18 to 20 mg. were used. Since intersolubility is an important factor in this separation, a certain relationship exists between the amount of mixed fatty acids separated and the volume of alcohol from which the lead soaps of the solid fatty acids are precipitated and in which the liquid acids are dissolved. At the time that these analyses were made, there was no method by which the volume could be satisfactorily adjusted for small amounts. As a result, in some cases no solid fraction precipitated out, while in others it was abnormally small. The converse was true when the mixed fatty acid fraction was larger than expected—the solid fraction was contaminated with liquid acids. Sinclair (personal communication) has since devised a satisfactory scheme whereby a constant proportion always exists between the mg. of fatty acids to be separated and the cc. of alcohol. The use of very small samples, however, is still not advisable.

All these factors tend to influence both the distribution and the iodine numbers of the solid and liquid acids and this fact must be kept in mind when drawing any conclusions from these analyses.

Since there were no appreciable differences between the muscles of the exercised and unexercised guinea pigs and the flying and non-flying pigeons, they were treated as single groups in the analysis of the data.

The values in general lead one to conclude that the ratio of liquid to solid acids is quite constant from muscle to muscle and animal to animal. On the whole, the averaged percentage of the liquid acids of the total fatty acids in muscle is 73 and that of the solid acids 27, as compared with a ratio of 60:40 in liver found by

Snider and Bloor (18) and Klenk and von Schoenebeck (19) in beef, and Sinclair (10) in the rat.

The iodine numbers of the liquid fatty acids in general were quite constant at about 173 for most of the muscles. On the other hand, definitely low iodine numbers were found for the heart muscle of the guinea pig, dog, and hen. For example, the value for the hen (Table III), which is typical for the other two animals, is 156 as compared with 175 for pigeon heart. These values were accompanied by a lower percentage of solid fatty acids, indicating a larger liquid acid fraction composed of fatty acids with a lower degree of unsaturation. Bloor (data unpublished) has recently found evidence of smaller amounts of arachidonic acid in heart muscle than is found in other muscles. The occasional wide differences in iodine numbers which were found in both the liquid and solid fractions might be caused by variation in the separation of these fractions, since, in general, the iodine numbers of the mixed fatty acids were more constant. Certain muscles of the hen and the pigeon had iodine values of the liquid fatty acid fraction as high as 188, but the mixed fatty acids were not unusually high. Only in the forearm of the pigeon (Table III) were there high values for both the mixed and liquid fatty acid fractions, namely 147 and 204, respectively.

DISCUSSION

The foregoing data show that, considered from the point of view of the nature of their fatty acids, the phospholipids in different muscles in a variety of animals are very similar if not identical, although the percentage amounts vary widely, apparently with the activity of the muscle. In liver, the degree of unsaturation of the liquid fatty acids and the percentage of solid fatty acids is higher than is found in muscle, a variation which can probably be attributed to differences in function, since liver phospholipid is largely metabolic, while that of muscle is essentially structural.

The small variations in the iodine numbers observed between the hen and pigeon, the wild and laboratory rabbit, and the cat and the dog may probably be attributed to diet, since the fat of the food has been shown to influence the fatty acids of the phospholipid (8-10). On the other hand, the general constancy of the iodine numbers might be interpreted as supporting to some degree the

results of Terroine and Bélin (20) who found that the iodine numbers of the fatty acids of the *élément constant* (largely phospholipid fatty acids) were constant.

SUMMARY

The nature of the fatty acids found in the phospholipid of muscle as shown by the ratio of saturated to unsaturated acids and by the iodine number of the unsaturated acids was found to be quite constant over a considerable spread of muscles in a variety of animals both exercised and unexercised.

As it stands at present, the effect of exercise is not reflected in the degree of unsaturation of the component fatty acids of muscle phospholipid.

The author wishes to express her indebtedness to Professor W. R. Bloor for his advice and criticism throughout the course of this study.

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A CONVENIENT METHOD FOR THE PREPARATION OF GALAC YEAST

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Galac yeast may be defined as a yeast which has acquired the property of fermenting galactose as a result of special treatment. This term was originated by Harding (1). All yeasts do not require acclimatization in order to ferment galactose, *e.g.* *Saccharomyces marxianus*, but ordinary yeasts do. Slator (2) was one of the early workers to convert a yeast which was not capable of fermenting galactose into what we now call galac yeast. Ordinary yeast of the so called bakers' type may be converted to galac yeast. Recently Harding and coworkers (1, 3) have suggested that galac yeast might be used in studies on the galactose content of biological fluids such as blood and urine. We have had the pleasure of preparing several samples of galac yeast for the late Dr. Harding, which he reported (3) to be very satisfactory with respect to galactose removal power. Numerous requests from other workers for galac yeast have shown that there is considerable interest in the problems studied by Harding *et al.*

The galac yeast supplied to Harding was prepared by a process which required special technique and equipment not available in most laboratories. The purpose of this paper is to give a convenient and inexpensive method for making small quantities of galac yeast, which does not involve special equipment. This method depends on the growth of a pure culture of yeast in a suitable medium which contains galactose. The cheapest source of galactose is ordinary milk sugar.

Hydrolysis of Lactose

Dissolve 500 gm. of milk sugar (U.S.P.) in a solution of 25 cc. of H_2SO_4 (sp. gr. 1.84) in 1250 cc. of water. Heat to a boil and allow

to simmer for 2 hours. Neutralize the hot solution with a thin paste of CaCO_3 . About 50 gm. of CaCO_3 are needed. The neutralization is carried to a reaction alkaline to Congo red but acid to litmus. Cool, and filter with suction through a thin layer of active carbon which is prepared on a Buchner funnel. Wash the filter cake with a little water (100 cc.) and make the combined, clear filtrates to 2000 cc. Thus 400 cc. are equivalent to 100 gm. of milk sugar. This solution may be sterilized and used as needed.

Preparation of Galac Medium

To 200 cc. of the hydrolyzed lactose solution add 15 gm. of yeast extract (Difco, dehydrated) or an equivalent yeast extract, and then make the volume to 1000 cc. The pH of a medium made in this manner was 6.1. After sterilization in flowing steam for 30 minutes on 2 successive days the pH was 5.4. The initial pH (before sterilization) should be adjusted to about 6.0 if necessary. We find it convenient to sterilize this medium in both test-tubes and 4 liter Erlenmeyer flasks. Each 4 liter flask should contain no more than 1 liter.

Growth of Galac Yeast

A pure culture of a bakers' type of yeast is used to inoculate one of the test-tubes of galac medium. At the end of 48 hours of incubation at 30° the yeast will have finished growing. The contents of the tube are then well shaken and transferred to 1 liter of sterile galac medium contained in a 4 liter flask. Another 48 hours incubation at 30° and the galac yeast is ready for use. Filtration of the yeast is best accomplished by suction on an 18.5 cm. Buchner funnel with a No. 50 Whatman paper. A crop of 10 gm. of moist yeast, of a consistency like compressed yeast, was obtained by the above method. Several batches of the yeast may be made at one time and filtered on the same funnel if more yeast is desired. The galac yeast is washed by suspending it in distilled water and again filtering. If more convenient a centrifuge may be employed to harvest and wash the yeast.

Galactose Fermenting Power

Yeast prepared in the above manner was found to compare favorably in galactose removal power with the yeast which was

supplied to the Toronto workers. 3 gm. of galac yeast will ferment 1 to 2 gm. of galactose in 3 to 4 hours at 30°, from 100 cc. of a 3 per cent galactose solution containing 1.5 gm. of monoammonium phosphate and 0.6 gm. of diammonium phosphate.

If stored at 2–5°, galac yeast will retain its fermenting activity for several weeks.

The method which we have described is not necessarily the best method of preparing galac yeast but it is, we hope, one that may prove useful because of its simplicity.

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THE MAGNESIUM-ACTIVATED LEUCYL PEPTIDASE OF ANIMAL EREPSIN*

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It is commonly assumed that the chief polypeptide-splitting enzyme of animal erepsin is the aminopolypeptidase studied by Waldschmidt-Leitz and his associates (1-3). This peptidase is usually estimated in erepsin preparations by the use of leucylglycine as test substrate, since this peptide is believed to be attacked by no other component of the erepsin system. Linderstrøm-Lang (4, 5), however, has published excellent evidence for the existence in erepsin of another enzyme, called by him "leucyl peptidase," which hydrolyzes both leucyldiglycine and leucylglycine rapidly. Linderstrøm-Lang's results, however, have not been accepted by other workers (6, 7). Data are presented in the present paper to show (a) that the leucyl peptidase of Linderstrøm-Lang, rather than the aminopolypeptidase of Waldschmidt-Leitz, is the chief leucyldiglycine-splitting enzyme of erepsin; (b) that this enzyme may be readily prepared, in good yields, free from aminopolypeptidase, from crude erepsin; and (c) that this enzyme is active only in the presence of magnesium ions.

EXPERIMENTAL

Methods

Erepsin was prepared by suspending hog duodenal mucosa in 4 times its weight of 85 per cent glycerol. The liquid obtained by straining such a preparation through cheese-cloth will be referred to as *glycerol extract*. When *glycerol extract* is treated with an

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equal volume of 0.06 N acetic acid (2), and the resulting precipitate centrifuged off, a clear solution is obtained which will be referred to as *acetic acid erepsin*.

Aminopolypeptidase was prepared by the method of Waldschmidt-Leitz and Balls (2). The only adsorbent which gave good results was monohydrated yellow ferric oxide ($\beta\text{-Fe}_2\text{O}_3 \cdot \text{H}_2\text{O}$) prepared by the method of Tommasi (8).

Peptidase determinations were made by adding 1 cc. of appropriately diluted enzyme solution to 2 cc. of substrate solution. 1 cc. samples of this mixture were titrated before and after incuba-

TABLE I
Composition of Substrates Used in Peptidase Determinations

Peptide	Peptide concentration	NaOH concentration	MgCl ₂ concentration	pH
	<i>M per l.</i>	<i>M per l.</i>	<i>M per l.</i>	
<i>dl</i> -Leucyldiglycine*.....	0.1	0.084	0.005	8.7
<i>dl</i> -Leucylglycine*.....	0.1	0.05	0.005	8.5
Triglycine†.....	0.05	0.025	0.0	8.0
Diglycine†.....	0.05	0.025	0.0	8.5
<i>dl</i> -Alanyldiglycine*.....	0.1	0.06	0.005	8.15
<i>dl</i> -Alanylglycine.....	0.1	0.05	0.005	8.3

2 cc. of the above substrates were diluted with 1 cc. of enzyme solution for determination.

* Linear hydrolysis: $E = 0.6x/t$, where E is the number of enzyme units present, x is the increase in titration (cc. of $M/15$ HCl) of a 1 cc. aliquot, and t the incubation time in minutes.

† Pseudomonomolecular hydrolysis: $E = \log(a/(a - x)) / 2t$ where a is the titration increase corresponding to total hydrolysis.

tion at 40°. The incubation time was usually 1 hour. Titrations were made in 90 per cent acetone with $M/15$ HCl, according to Linderstrøm-Lang (9). From the titration difference, the number of enzyme units present was calculated. These enzyme units are comparable with those used in a previous study (10). The composition of the substrates used and the method of calculation employed are given in Table I. MgCl_2 is present in the substrates attackable by leucyl peptidase.

Leucyl Peptidase—Crude erepsin hydrolyzes both leucyldiglycine and triglycine readily. If, however, crude erepsin is precipitated

with acetone and then with alcohol, the resulting preparation retains most of its leucyldiglycine activity, but its triglycine activity is practically entirely lost. An example of such a preparation will be given.

50 cc. of *glycerol extract* were diluted with 50 cc. of water, and 200 cc. of acetone were added. The precipitate was centrifuged off, suspended in 50 cc. of water, and clarified in the centrifuge. This solution still contained some triglycine activity. It was treated with 100 cc. of alcohol and 0.05 gm. of sodium acetate (the latter to assist flocculation). After 10 minutes, the precipitate

TABLE II
Activity Changes during Preparation of Leucyl Peptidase

Preparation	Substrate	Enzyme used	Incubation time	Titration increase	Activity
		cc.	min.	cc. <i>M/15</i>	<i>enzyme units per l.*</i>
I. Crude erepsin	Leucyldiglycine	0.250	60	0.296	11.8
	Alanyldiglycine	0.025	60	0.160	64
	Triglycine	0.250	60	0.340	15.1
II. Preparation I precipitated with acetone	Leucyldiglycine	0.09	60	0.202	11.2
	Alanyldiglycine	1.00	60	0.305	1.5
	Triglycine	1.00	60	0.035	0.1
III. Preparation II precipitated with alcohol	Leucyldiglycine	0.18	60	0.334	9.3
	Alanyldiglycine	1.00	60	0.234	1.2
	Triglycine	1.00	840	0.042	0.01

* Corrected for volume changes during manipulation.

was centrifuged off and resuspended in 50 cc. of water. Table II shows the effect of the above procedure on the activity of this preparation.

It is obvious that the leucyldiglycine activity of the preparation just described cannot be due to aminopolypeptidase, since the latter enzyme hydrolyzes triglycine very rapidly (2). The preparation of Table II, having no triglycine activity, must be free from aminopolypeptidase. The leucyldiglycine-splitting enzyme which is present will be referred to as leucyl peptidase, since it is probably identical with the enzyme postulated by Linderstrøm-Lang.

Aminopolypeptidase—The difference in activity between leucyl

peptidase and aminopolypeptidase will be more apparent if Table II is compared with Table III, which shows the activity toward several substrates of an aminopolypeptidase preparation, and of the *acetic acid erepsin* from which it was made. In Tables III to V, titration figures are omitted for purposes of brevity. It will be seen from Table III that less than 5 per cent of the leucyldiglycine activity of the starting material was recovered as aminopolypeptidase, while 38 per cent of the original triglycine activity was so recovered. It will also be noted that aminopolypeptidase attacks triglycine more than 5 times as rapidly as leucyldiglycine, whereas leucyl peptidase (Table II) attacks the latter more than 1000 times as rapidly as the former. Aminopolypeptidase splits alanyldi-

TABLE III
Activity of Crude Erepsin and of Aminopolypeptidase

Substrate	Activity	
	Acetic acid erepsin	Aminopoly-peptidase
	<i>units per l.</i>	<i>units per l.*</i>
Leucyldiglycine	12.7	0.58
Triglycine	9.3	3.5
Leucylglycine	17.9	0.04
Alanyldiglycine	39.2	7.1
Diglycine	2.1	0.06

* Corrected for volume changes during manipulation.

glycine more than 10 times as rapidly as leucyldiglycine, while leucyl peptidase attacks the alanyl peptide relatively slowly. From Tables II and III it is apparent that the chief leucyldiglycine-splitting enzyme of erepsin is not aminopolypeptidase, but the enzyme here called leucyl peptidase.

Magnesium Activation of Leucyl Peptidase—The presence of small concentrations of magnesium salts has no effect on the activity of aminopolypeptidase. The activity of leucyl peptidase, however, is a function of magnesium ion concentration. A quantitative study of the magnesium activation of leucyl peptidase gave the results shown in Fig. 1. The effect of added magnesium on the activity of a leucyl peptidase preparation toward leucyldiglycine is indicated by the points of Curve 1. These data suggested

the existence of a reversible reaction between enzyme and magnesium. A leucyl peptidase preparation was therefore given a short dialysis to reduce its magnesium content. The effect of added magnesium on leucyldiglycine hydrolysis by this preparation is shown by the points of Curve 2. Here the activity when no magnesium was added was very low, and it was therefore possible by means of graphic extrapolation to estimate the ionized magnesium content of the enzyme preparation itself, and, correcting for this, to obtain a value of 0.0018 for the dissociation constant

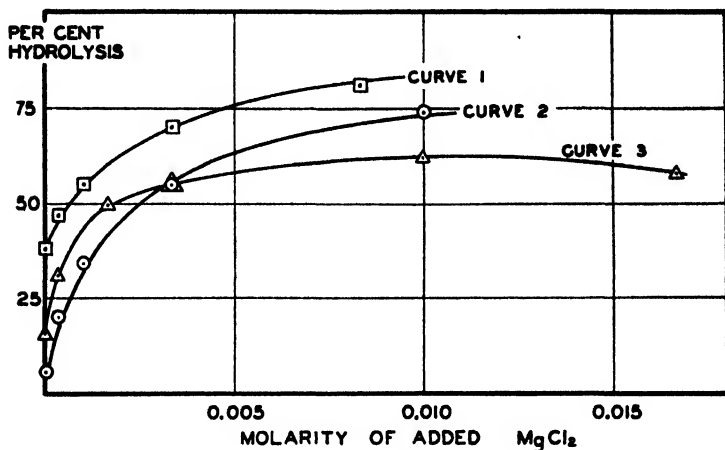


FIG. 1. Effect of magnesium concentration on the activity of leucyl peptidase. Curve 1, leucyl peptidase, leucyldiglycine substrate. Points experimental; solid curve calculated. Curve 2, dialyzed leucyl peptidase, leucyldiglycine substrate. Points experimental; solid curve calculated. Curve 3, dialyzed leucyl peptidase, alanyldiglycine substrate.

of the magnesium-enzyme complex. The solid line of Curve 2 is a theoretical curve constructed on the basis of this dissociation constant. All the experimental points fall very close to it. The dissociation constant known, it was possible to determine algebraically the ionized magnesium content of the enzyme preparation used in obtaining the data of Curve 1 and to construct, on the basis of the same dissociation constant, the theoretical curve represented by the solid line of Curve 1. In this case also the experimental data indicate the existence of a reversible reaction

involving magnesium. It is therefore probable that the magnesium ion forms a reversibly dissociable compound with the enzyme-substrate complex, or possibly with the free enzyme, and that only enzyme in combination with magnesium is hydrolytically active.

Curve 3 shows the effect of magnesium concentration on alanyldiglycine hydrolysis by a dialyzed leucyl peptidase preparation. Here the very apparent inhibitory effect of the higher magnesium concentrations makes the determination of a dissociation constant difficult.

Leucyl peptidase is thus the only known peptidase requiring a specific activator. That magnesium plays a part in the activity of other peptidases is, however, very probable. Aminopolypeptidase preparations which have been partially inactivated by dialysis show a slight but definite increase in activity upon standing in the presence of magnesium ions. Moreover, preliminary experiments have shown that the rate of hydrolysis of leucyldiglycine by the yeast peptidase system is doubled in the presence of 0.003 M $MgCl_2$.

pH Optimum and Kinetics of Leucyl Peptidase and Aminopolypeptidase—In Fig. 2 are plotted pH-activity curves for the hydrolysis of peptides by leucyl peptidase and aminopolypeptidase. In all cases the substrate concentrations were those of Table I. The enzyme concentration was so chosen that in each case sufficient enzyme was present to give 60 to 75 per cent hydrolysis of the substrate at the optimum pH. No buffer, aside from the substrate itself, was used. When a tripeptide (pK_a , about 8.0) is hydrolyzed to a dipeptide (pK_a , about 8.3) and an amino acid (pK_a , about 11.7) very little pH change occurs. Glass electrode pH determinations were made in each case at the end of the hydrolysis period.

In the case of aminopolypeptidase, it will be noted that the curves for alanyldiglycine and triglycine are very similar, whereas the curve for leucyldiglycine shows a less sharp pH optimum, probably because of the exceedingly high enzyme affinity of this substrate. It will be seen that aminopolypeptidase is fairly active at pH 6, where 99 per cent of the substrate is present as zwitter ion.

The most striking feature of the curves for leucyl peptidase is the sharp break at pH 9.2. This break is always observed, and

is due to precipitation of $\text{Mg}(\text{OH})_2$. The solubility product of $\text{Mg}(\text{OH})_2$ is 1.2×10^{-11} , and the Mg concentration of the enzyme-

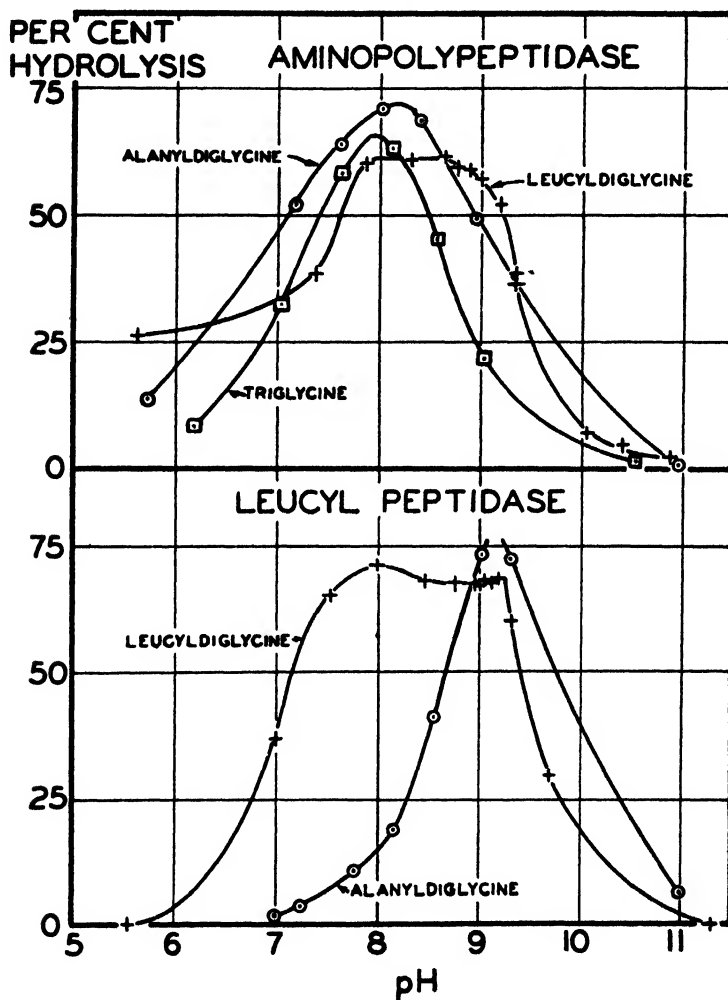


FIG. 2. Variation of leucyl peptidase and aminopolypeptidase activity with pH.

substrate mixture was 0.0033 M. Therefore the solubility product should be reached at pH 9.2, which is the pH value at which the

activity of leucyl peptidase begins rapidly to decrease. The decreased activity at pH values above 9.2 is thus due to a removal of activator from solution. Another property of leucyl peptidase is apparent from the curves. This enzyme, unlike aminopolypeptidase, is unable to attack the zwitter ion form of the substrate. Hydrolysis was never obtained at pH values where only inappreciable amounts of peptide anion were present. The broad pH optimum of leucyldiglycine hydrolysis is probably here also due to high enzyme-substrate affinity.

The kinetics of hydrolysis of leucyldiglycine, alanyldiglycine, and triglycine have been studied. The conditions were those of the peptidase determinations, as given in Table I. Space will not be taken to tabulate the data. It was found that leucyldiglycine hydrolysis by both leucyl peptidase and aminopolypeptidase proceeded linearly up to at least 90 per cent hydrolysis. Alanyldiglycine hydrolysis was linear up to approximately 75 per cent for both enzymes. In the case of this peptide, the purity of the enzyme preparation made considerable difference in the shape of the hydrolysis curve. Triglycine hydrolysis was always pseudomonomolecular.

Specificity of Leucyl Peptidase and Aminopolypeptidase—In Table IV are given data obtained in an experiment in which the comparative activity of leucyl peptidase and aminopolypeptidase toward a number of substrates was determined. While leucyl peptidase does not attack polyglycines, it attacks glycyllaucylglycine readily, showing that the presence of a glycyl group in the amino position does not prevent hydrolysis. If the free amino group of leucyldiglycine is methylated, hydrolysis is prevented, showing the essential nature of the intact amino group. Leucyl peptidase does, however, attack the decarboxylated dipeptide leucylmethylamine, showing that the carboxyl group need not be present. Linderstrøm-Lang (11) correctly ascribed the hydrolysis of this compound by crude crepsin to leucyl peptidase. Aminopolypeptidase, as may be seen, does not attack the decarboxylated dipeptide glycyllmethylamine, but does hydrolyze the methylated tripeptide sarcosyldiglycine. Every aminopolypeptidase preparation we have investigated has shown ready hydrolysis of this compound. It is not known whether the hydrolysis is due to aminopolypeptidase itself, or to another enzyme always present in

our preparations. However, it has been found that sarcosyldiglycine is split to sarcosine and diglycine, thus ruling out the action of a carboxypeptidase. This point requires further investigation, since aminopeptidases presumably require an intact amino group. It might also be mentioned that as would be expected aminopolypeptidase splits alanyldiglycine to alanine and diglycine, and that leucyl peptidase splits leucyldiglycine to leucine and diglycine.

TABLE IV
Specificity Comparison of Peptidases

Substrate	Leucyl peptidase Hydrolysis of one linkage* in		Aminopolypeptidase Hydrolysis of one linkage* in		Mold amino- polypep- tidase
	1 hr.	5 hrs.	1 hr.	5 hrs.	
	per cent	per cent	per cent	per cent	
<i>dl</i> -Leucyldiglycine	39	101	6	32	+++
<i>dl</i> -N-Methylleucyldiglycine ..	0	0	0	0	—
Glycyl- <i>dl</i> -leucylglycine	38	194	28	100	++
<i>dl</i> -Alanyldiglycine	10	53	69	110	++
Triglycine	0	3	42	99	—
Tetraglycine	0	1	8	20	—
Sarcosyldiglycine	0	0	6	28	—
<i>dl</i> -Leucylmethylamine	6	10	0	0	+
Glycylmethylamine	0	0	0	0	—
<i>dl</i> -Leucylglycine	40	100	0	3	—

Substrates containing an asymmetric carbon atom were present in $m/15$ concentration, others $m/30$. The sparingly soluble glycyl-*dl*-leucylglycine was also present in $m/30$ concentration. The pH in all cases was 8.0. $m/300$ $MgCl_2$ was present in all cases. The enzyme concentration was also held constant.

* In the case of *dl* mixtures, per cent hydrolysis of one linkage of one component is indicated.

In the last column of Table IV is indicated, for comparison, the behavior of *Aspergillus parasiticus* aminopolypeptidase (10) toward the various substrates. Its specificity is remarkably like that of leucyl peptidase, but very different from that of ereptic aminopolypeptidase. This mold aminopolypeptidase is, however, not activatable by magnesium.

Dipeptidases—As may be seen from Table IV, leucyl peptidase preparations hydrolyze leucylglycine, a typical dipeptidase sub-

strate, rapidly. In Table V the dipeptidase activity of a leucyl peptidase preparation is compared with that of the *acetic acid erepsin* from which it was made. Tripeptide activities are also included for comparison. It will be noted that leucyl peptidase preparations behave in a similar manner toward dipeptides and corresponding tripeptides. That is, leucylglycine is split rapidly, alanylglycine slowly, and diglycine extremely slowly if at all. Moreover, dipeptide hydrolysis by leucyl peptidase preparations is also activatable by magnesium.

This parallelism in dipeptide and tripeptide specificity seems to indicate that we are dealing with a single enzyme, capable of

TABLE V
Dipeptidase Activity of Acetic Acid Erepsin and of Leucyl Peptidase

Substrate	Activity	
	Erepsin	Leucyl peptidase
	units per l.	units per l.*
Leucylglycine.....	11.1	6.1
Alanylglycine.....	18.4	0.52
Diglycine.....	0.5	0.02 \pm 0.02
Leucyldiglycine.....	9.4	6.1
Alanyldiglycine.....	32.4	1.0
Triglycine.....	6.5	0.02 \pm 0.02

* Corrected for volume changes during manipulation.

hydrolyzing both dipeptides and tripeptides. There are other facts which apparently support such an assumption. Leucyl peptidase preparations prepared during the course of the present investigation, some of them prepared by different methods, and some of them from mucosa from different sources, all exhibited a ratio of leucyldiglycine activity to leucylglycine activity of very nearly unity. Moreover, numerous attempts to prepare a dipeptidase-free leucyl polypeptidase or a polypeptidase-free leucyl dipeptidase have met with uniform failure. Furthermore, if leucyl peptidase is allowed to act upon a substrate solution containing both leucyldiglycine and leucylglycine, the total rate of hydrolysis is no greater than is obtained with either one of the substrates separately. Such data suggest that the leucylglycine-splitting

component of leucyl peptidase is identical with the leucyldiglycine-splitting component. On the other hand, there are data which seemingly indicate that the two are not identical. It has been possible by several means to produce very considerable displacement of the leucylglycine to leucyldiglycine activity ratio of many leucyl peptidase preparations. Up to the present it has not been possible to prove either the homogeneity or the non-homogeneity of our leucyl peptidase preparations.

A leucyl peptidase preparation, as may be seen from Table V, retains more than half of the leucylglycine activity of the crude erepsin from which it was made. It retains, however, very little of the alanyl-glycine activity of the crude erepsin, and none of the diglycine activity of the crude erepsin. It follows, of course, that erepsin contains a dipeptidase apart from the dipeptidase activity of leucyl peptidase. The enzyme responsible for all of the diglycine activity, for most of the alanyl-glycine activity, and undoubtedly for a part of the leucylglycine activity of crude erepsin may for the present be called glycyl dipeptidase since it may be determined in the presence of leucyl dipeptidase by the use of diglycine as substrate. The aminopolypeptidase of Waldschmidt-Leitz may be called glycyl polypeptidase since triglycine is apparently a specific substrate for it. Glycyl dipeptidase hydrolyzes alanyl-glycine much more rapidly than diglycine, just as glycyl polypeptidase hydrolyzes alanyldiglycine more rapidly than triglycine.

During the course of the present investigation, the possibility was considered that the intestinal mucosa material being used was in some way different from that used by previous workers in the field. Accordingly, a sample of crude erepsin prepared in Professor Waldschmidt-Leitz's laboratory, with mucosa from the source used by Waldschmidt-Leitz and his associates, was investigated. The authors are very grateful to Professor Waldschmidt-Leitz and to Dr. Anton Schäffner for supplying this preparation. This crude erepsin, on adsorption with various adsorbents, and on acetone precipitation, gave results identical with those obtained with our preparations.

SUMMARY

The chief leucyldiglycine-hydrolyzing enzyme of hog erepsin is not the aminopolypeptidase of Waldschmidt-Leitz, which attacks

this substrate much more slowly than alanyldiglycine and triglycine. It is an enzyme probably identical with the leucyl peptidase postulated by Linderstrøm-Lang. This leucyl peptidase has been obtained free from aminopolypeptidase. It hydrolyzes leucyldiglycine, alanyldiglycine, and glycylleucylglycine, but not triglycine or tetraglycine. This peptidase is active only in the presence of magnesium ions. Leucyl peptidase is similar in specificity to the aminopolypeptidase of *Aspergillus parasiticus* which, however, is not activated by magnesium. All leucyl peptidase preparations thus far obtained show a magnesium-activatable hydrolysis of leucylglycine, but do not hydrolyze diglycine. Erepsin contains a dipeptidase distinct from the dipeptidase activity of leucyl peptidase preparations.

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STUDIES ON ANIMAL LIPIDS

XI. THE REINECKATE OF THE POLYDIAMINOPHOSPHATIDE FROM SPLEEN*

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In Paper III of this series Fränkel, Bielschowsky, and Thannhauser (1) describe a polydiaminophosphatide isolated from fresh pig liver. These authors assume for this crystalline substance the constitution of a trimeric sphingomyelin, based on quantitative determinations of fatty acid split off from this substance. They suggest a formula for this polydiaminophosphatide, which combines the three sphingomyelins in such a way that the choline of the one sphingomyelin is tied up with the phosphoric acid group of the other, forming an ester. This formula was hypothetical and was meant to serve as a suggestion for further investigation. Papers published in the meantime contest not only the accuracy of this formula (Klenk (2)) but also the existence of a polymeric sphingomyelin (Fischgold and Chain (3)). Concerning the formula, it is to be said that we never considered it as proved. In fact, we are as yet unable to establish either this or any other formula for the polydiaminophosphatide as correct. On the other hand, the doubt which Fischgold and Chain cast upon the existence of a polydiaminophosphatide based on titration of some one sphingomyelin may be dispelled by this paper dealing with a crystalline salt of the polydiaminophosphatide whose analysis is clearly consistent with our assumption of a trimeric sphingomyelin.

It was found that bovine spleen is a better starting material than pig liver. The preparation is made in essentially the same manner as for the preparation and yield of the trimeric sphingo-

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myelin (polydiaminophosphatide), from pig liver. We have succeeded in separating the contaminating substances containing sugar in a simple and rapid manner using the principle of chromatographic adsorption analysis. Formerly it was necessary to recrystallize the polydiaminophosphatide several times, with great waste of material. For the chromatographic adsorption the lipid material is dissolved in methanol-petroleum ether 1:10 and passed through a glass tube containing aluminum oxide (Merck, according to Brockmann). The preparation obtained by this procedure is colorless. It gives an entirely negative Molisch reaction.

Exploring systematically those reagents which might be expected to precipitate diaminophosphatides, we found that Reinecke acid is a reagent which precipitates the polydiaminophosphatide completely. On the other hand, the monoaminophosphatides lecithin and cephalin, and the cerebrosides and ceramides are not precipitated by Reinecke acid. The Reineckate of the polydiaminophosphatide is formed by adding a solution of Reinecke salt in methanol to a solution of polydiaminophosphatide in methanol previously rendered acid by a few drops of concentrated HCl. This Reineckate is a pink crystalline powder slightly soluble in cold methanol but insoluble in cold acetone and ether. It is sensitive to light and must be protected by storing in brown flasks. On heating in a glass capillary it soon becomes greenish and beyond 150° it decomposes completely. The lipid is regenerated from the Reineckate by dissolving the Reineckate in hot methanol and precipitating the Reinecke acid as silver salt in the hot, saturated methanol solution. The silver surplus in the solution is precipitated with H₂S; the filtrate is concentrated and precipitated by acetone.

It is somewhat difficult to obtain exact analytical figures of the Reineckate because the substance is slightly decomposed on recrystallizing from hot methanol. Because of this observation we analyzed the Reineckate in the state in which it was originally crystallized from a pure polydiaminophosphatide. The results of the analysis are as follows:

Reineckate of Polydiaminophosphatide from Spleen—
3.168 mg. substance: 7.09 mg. CO₂, 2.99 mg. H₂O
4.181 " " : 0.220 cc. N₂, 763 mm., 31.5°

*Reineckate of Polydiaminophosphatide from Liver—*2.991 mg. substance: 6.68 mg. CO₂, 2.88 mg. H₂O7.020 " " : 0.377 cc. N₂, 763 mm., 32°*Reineckate of Polydiaminophosphatide—*C₁₃₁H₂₆₅N₁₂P₃O₁₅S₄Cr. Calculated. C 60.9, H 10.3, N 6.5

Spleen. Found. " 61.0, " 10.56, " 5.92

Liver. " " 60.91, " 10.77, " 6.03

*Theoretical Values for Reineckate of Monomeric Sphingomyelins—*Palmitylsphingomyelin. C₄₃H₈₇N₃PO₇S₄Cr. C 49.7, H 8.47, N 10.8Stearyl sphingomyelin. C₄₅H₉₁N₃PO₇S₄Cr. " 50.7, " 8.7, " 10.5Lignoceryl sphingomyelin. C₅₁H₁₀₃N₃PO₇S₄Cr. " 53.2, " 9.03, " 9.72

The results of our analyses show conclusively that in our Reineckate sphingomyelin and the Reinecke acid radical are in a ratio of 3:1. Therefore, 3 molecules of monomeric sphingomyelins are combined with one Reinecke acid radical or 1 molecule of an already preformed trimeric sphingomyelin; a polydiaminophosphatide reacts with 1 molecule of Reinecke acid.

In accordance with the analogy of the Reineckate of choline and different choline derivatives we believe we are justified in assuming that the latter constitution of the Reineckate is correct. The analysis of the Reineckate, therefore, may be considered as important support of the previous assumption of the trimeric nature of our polydiaminophosphatide.

The use of the Reineckate of the polydiaminophosphatide gives evidence not only of the size of the lipid molecule but also can serve as an accurate means for determining quantitatively the phospholipids in animal organs. Since only diaminophosphatides and no monoaminophosphatides give insoluble compounds with Reinecke acid in cold methanol, it is now possible with the aid of this reagent to determine both groups of phospholipids simultaneously present with greater accuracy than had previously been possible. Such an application of the Reineckate for quantitative lipid analysis of the blood serum and stromata of the red blood cells will be described in a subsequent report.

Preparation of Diaminophosphatide from Spleen—30 kilos of bovine spleen, as fresh as possible, are freed from fat and connective tissue and ground in a meat grinder. The hashed organ is mixed with an equal amount of acetone, left for 48 hours, sucked dry, again treated with the same amount of fresh acetone, and

again sucked dry. The coarse organ powder is dried completely by heating not higher than 50° in a vacuum dryer. The material is ground as fine as possible, mixed with Raschig rings and extracted for 24 hours with ether in the huge extraction apparatus described by former coworkers Fränkel and Pollanz (4). The extract is concentrated to a volume of 3 liters and put in an ice box for 3 to 5 days. The precipitate formed during this period is centrifuged, washed in the centrifuge three times with ether and three times with acetone, and dried in a vacuum desiccator. The dried substance is ground with $1\frac{1}{2}$ times the amount of sea sand (sea sand washed and ashed, Merck) and extracted in a Soxhlet apparatus with ether. The extraction is complete when lignoceryl-sphingosine does not further separate from the solution. This extraction requires 3 or 4 days. The content of the thimble is suspended in petroleum ether in an Erlenmeyer flask. Methanol is added until the substance is dissolved and sand remains undissolved. The methanol-petroleum ether mixture is about 1:10. The sand is filtered and carefully washed with a methanol-petroleum ether mixture of 1:10. The methanol-petroleum ether solution is filtered through a glass tube containing Al_2O_3 (Merck, according to Brockmann) according to the prescription of the chromatographic analysis of Winterstein and Stein (5). The filtrate is clear and colorless. The solution is concentrated to a small volume. The polydiaminophosphatide is completely precipitated with acetone. It is sucked dry and recrystallized from chloroform-ethyl acetate.

Reineckate of Polydiaminophosphatide—The polydiaminophosphatide is dissolved in just enough methanol, a few drops of concentrated HCl are added to this solution, and to this a freshly prepared solution of Reinecke salt in methanol is added. The precipitate is formed quickly. After standing in the ice box the precipitate is filtered through a porous crucible, washed with a small amount of ice-cooled methanol and ether, and dried.

Decomposition of Reineckate—The Reineckate is dissolved in hot methanol to which a solution of silver nitrate in methanol is added. The silver Reineckate precipitated is filtered and H_2S passed through the colorless solution. Silver sulfide is filtered and this solution is evaporated to a volume as small as possible. The

polydiaminophosphatide is precipitated with acetone, sucked dry, and recrystallized from chloroform-ethyl acetate.

SUMMARY

1. Crystalline Reineckate of polydiaminophosphatide is described and its significance to the polymeric structure of the polydiaminophosphatide is discussed.

2. It is shown that it is possible to separate monoaminophosphatide and diaminophosphatide by precipitating the latter as Reineckate.

3. A description of the preparation of polydiaminophosphatide from bovine spleen is given.

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STUDIES ON ANIMAL LIPIDS

XII. A METHOD FOR QUANTITATIVE DETERMINATION OF DIAMINOPHOSPHATIDE IN ORGANS AND FLUIDS. APPLICATION TO STROMATA OF RED BLOOD CELLS AND SERUM*

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Lipids act differently toward water and solvents containing water. Four groups may be distinguished with regard to their behavior toward water: the sterols, the cerebrosides, the diaminophosphatides, and the monoaminophosphatides. At the beginning of this series the sterols are to be placed as the most hydrophobic; at the end of the series the monoaminophosphatides are to be placed as the most hydrophilic lipids. It is very likely that these different reactions toward water play an important part in the quantitative relation of the different lipids present in the organs and in the blood.

Therefore, it would be of great value to have quantitative methods to determine these four lipid groups so as to study the physiological and pathological changes of the quantitative proportion of the particular lipid present in the lipid mixture of cells and fluid. To date there are only good methods to determine sterols, but the group of phosphatides was solely reckoned from total P determinations. By so doing monoamino- and diaminophosphatides were shown together in spite of their different behavior toward water and despite their different chemical constitution indicating a different biological task and effect in the organism.

The Reineckate of the diaminophosphatide complex described in the previous paper (1) enables us (1) to separate mono- and diaminophosphatides; (2) to evaluate the amounts of diamino-

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and monoaminophosphatides from the results of the analysis of the Reineckate and from total P determination in organs and fluids; (3) to bring also the other N-containing compounds (cerebrosides and ceramides) within the sphere of analytical determination.

Procedure

Organs such as spleen, liver, and brain are dried by freezing in an ether-solid CO_2 mixture. The frozen pieces of organ are then dried in a vacuum desiccator over P_2O_5 .

A sufficient amount of stromata (1 to 3 gm.) or serum (50 cc.) to be analyzed is dried as much as possible in a stream of warm air (electric fan) and afterwards completely dried in a vacuum desiccator over P_2O_5 . We have found this simple way of drying the material to be the most satisfactory. If the material is dried in a higher temperature, there is danger of decomposing the diamino-phosphatides. Drying with gypsum and other inorganic dehydrating agents has disadvantages: (1) the following extraction is incomplete; (2) the volume of the material to be extracted would be too large.

After complete drying, the weighed organs (fluids are measured volumetrically) are ground to a fine powder with 6 to 8 times their volume of very fine sea sand (Merck) and extracted. The sea sand serves to transport the ground material quantitatively into the thimble and to prevent the dried material from caking. 40 cc. of a mixture of methanol and chloroform (1:1) are used as a solvent for the extraction. In order to obtain complete extraction the thimble is placed in the apparatus so that the thimble is suspended only 2 cm. above the boiling solvent. A Soxhlet extraction apparatus proved in this case to give incomplete extraction. We use a special long neck, round bottom flask with a ground-in reflux condenser. The extraction runs for at least 12 hours. The cold extract is filtered into a 50 cc. volumetric flask and an aliquot part is used for the different determinations.

In comparison with the extraction according to Bloor (2), with a mixture of ethanol and ether, the described procedure has the advantage that only a small amount of solvent is necessary. To extract 50 cc. of serum 1000 cc. of Bloor's solvent are necessary, whereas we are able to extract the same amount of dried serum

with only 40 cc. of chloroform-methanol mixture. Besides the economy of the solvent in our extraction, it is of greater importance because we find too small values of diaminophosphatide when a greater volume of ethanol-ether mixture which is to be concentrated in a vacuum is used. This difference may be explained by observing that polydiaminophosphatide is partly volatile with vaporized ethanol in a vacuum. Thus we have proved in duplicate determinations—one with Bloor's solution and one with chloroform-methanol (1:1)—that the total lipid phosphorus is the same, but we found only one-third of the amount of diaminophosphatides which we found with chloroform-methanol. That indicates that the extractions with ethanol-ether and with chloroform-methanol are equally good, but there is some loss of diaminophosphatide through concentration of the alcoholic solution in a vacuum by volatilizing polydiaminophosphatide with vaporized ethanol.

The 50 cc. of methanol-chloroform extract are used in the following manner.

Determination of Diaminophosphatide—25 cc. of the extract are evaporated in a 50 cc. Erlenmeyer flask, on a water bath, to dryness. The residue is extracted with a little hot methanol several times and the hot methanol solution is filtered in a 25 cc. Erlenmeyer flask. A turbidity in the filtrate is clarified by slight warming on the water bath. 3 drops of concentrated HCl are added and 10 cc. of a saturated, freshly prepared solution of Reinecke salt in methanol. The mixture is allowed to cool to room temperature and afterwards put in the ice box overnight. The precipitate is filtered through a porous, porcelain crucible which is completely surrounded by ice in an adapted glass container, washed with a little ice-cold methanol a few times, and finally very carefully washed with ether to remove traces of cholesterol. The crucible is dried in a vacuum desiccator to a constant weight by multiplying the weight of the precipitate by 0.877, and the amount of the diaminophosphatide present in 25 cc. of extract is obtained directly. This factor, 0.877, is calculated from the relation of the molecular weight of the polydiaminophosphatide, 2266, to the molecular weight of its Reineckate, 2584. It is possible to determine as low as 20 mg. of polydiaminophosphatide.

Using the factor mentioned above we assume that the precipitate

is only formed by the Reineckate of polydiaminophosphatide. We believe we are justified in the above calculation because we have pointed out in the previous paper that only polydiaminophosphatide and not monoaminophosphatide and cerebroside give an insoluble Reineckate in cold methanol. The lipids used for these experiments have been purified as much as possible.

In the following experiments dealing with the amount of polydiaminophosphatide in the blood serum, we observed that the Reineckate precipitated from the serum and stroma is partly soluble in cold acetone. It is possible to separate the Reineckate into an acetone-soluble and into an acetone-insoluble part. The Reineckate of the polydiaminophosphatide is, as described in the previous paper, insoluble in cold acetone, so that the observation of an acetone-soluble Reineckate indicates another lipid present in the serum and stroma which gives an acetone-soluble Reineckate.

To clarify this phenomenon, we prepared about 1 gm. of Reineckate from 1000 cc. of bovine serum in the manner described above. This Reineckate is finely ground and digested with acetone at room temperature as long as the solution shows any red color. By this procedure almost equal amounts of two different Reineckates are obtained, which are denoted further as acetone-soluble and acetone-insoluble Reineckate. In both, N and P were determined.

Acetone-Soluble Reineckate—

4.728 mg. substance: 0.451 cc. N_2 , 28° , 7.62 mm. Hg

8.737 " " : 12.37 mg. precipitate, factor 0.014524

Reineckate of stearyl sphingomyelin.

$C_{46}H_{91}N_2PO_7S_4Cr$. Calculated. N 10.5, P 2.91

Acetone-soluble Reineckate. Found. " 10.86, " 2.06

From the acetone-soluble Reineckate the lipid was recovered. The small amount obtained could not be further purified. The analysis showed N 2.35, P 2.82, N:P 1:1.84. The acetone-insoluble Reineckate corresponds with the Reineckate of a polydiaminophosphatide described in the previous paper (N 6.50, P 4.11). The acetone-soluble Reineckate analyzed above shows a higher value for N (10.86) and a lower value for P (2.06) according to a Reineckate of a monomeric diaminophosphatide (stearyl sphingomyelin) whose theoretical values are N 10.5 and P 2.91. When we consider these analytical figures in regard to the ratio

N:P of the lipid recovered from the acetone-soluble Reineckate, which does not vary very much from 2, the assumption may be justified that the acetone-soluble Reineckate of the serum is a Reineckate of a monomeric diaminophosphatide (sphingomyelin).

It will be the subject of further investigation to clarify this question. We have already discussed this problem because the weight of the Reineckate obtained in our lipid analysis may in the future be evaluated separately in an acetone-soluble and acetone-insoluble part. Up to now we have calculated the total amount of Reineckate of polydiaminophosphatide, by multiplying by the factor 0.877, according to the molecular composition of the polydiaminophosphatide Reineckate. In the future, if the assumption discussed above, that the acetone-soluble Reineckate is proved the Reineckate of a monomeric diaminophosphatide, we will have to calculate the weight of the acetone-soluble and acetone-insoluble Reineckate separately by multiplying the acetone-insoluble by the old factor 0.877 and the acetone-soluble by a factor calculated as an average from a Reineckate which is composed of a monomeric diaminophosphatide and 1 molecule of Reinecke acid (see previous paper).

Determination of Total Phosphorus and Total Phospholipid—5 cc. of the original extract are evaporated to dryness in a small Kjeldahl flask; the residue is digested with 2 cc. of fuming sulfuric acid and afterward made up to 50 cc. The determination of phosphorus is carried out according to the colorimetric method of Briggs, an aliquot part (5 cc.) of this latter solution being taken. By multiplying the mg. per cent of phosphorus by 25, the total amount of phospholipids is obtained.

Determination of Total Nitrogen—The total N, according to the micromethod of Parnas, is determined in an aliquot part of the solution used above for total phosphorus determination.

Application of This Method to Determine the Diaminophosphatides and Total Phospholipids in Stromata of Red Blood Cells

Preparation of Dry Stromata—Approximately 150 cc. of oxalated blood are centrifuged half an hour, the plasma is poured off, and the remaining red cells are washed three times with normal saline. The washed red cells are hemolyzed by adding distilled water to an excess of 15 times the original blood volume. This solution is

filtered through a layer of cotton (about 3 inches in thickness); the stromata are precipitated in the filtrate by passing CO_2 through the solution for about 5 minutes. The precipitated solution is allowed to stand overnight in the ice box, the supernatant liquid is syphoned off as much as possible, and the precipitate of stromata is washed by centrifuging with distilled water as long as any red color remains in the water. The remaining paste-like stromata are dried in a flat glass dish by passing a stream of warm air over it, a simple hair dryer or a vacuum dryer which is heatable being used. The dried stromata are scraped from the glass dish and dried to constant weight in a vacuum desiccator. The results of the analysis are calculated on the weight of dried stromata.

Extraction—The weighed stromata (at least 1 gm. equivalent to 150 to 200 cc. of blood) are ground to a fine powder and mixed intimately with 6 to 8 times their volume of fine sea sand (washed and ashed, Merck). Enough from this latter amount of sea sand is kept to clean the mortar quantitatively of the applied material. The mixture is transferred to a thimble (80×22 mm., Schleicher) and extracted with 40 cc. of a chloroform-methanol 1:1 mixture for at least 12 hours. It is very important for complete extraction that the thimble is fixed in a long neck, round bottom flask 2 cm. above the liquid. The extract is filtered into a 50 cc. volumetric flask and made up to volume with chloroform-methanol (1:1). The determinations are carried out with aliquot parts as described above.

The normal figures for diaminophosphatides in stromata are between 4 and 6 mg. per cent. If the difference of total phospholipids to the amount of diaminophosphatides found by gravimetric analysis is evaluated as monoaminophosphatides, we find 5 to 11 mg. per cent present as monoaminophosphatides (lecithin and cephalin) in the stromata of red blood cells (Table I). The ratio of diamino- to monoaminophosphatides in stromata is, according to these figures, 1:1 to 1:2. The ratio of diaminophosphatides to total phospholipids is 1:2 to 1:3.

Quantitative Determination of Diaminophosphatides in Serum

At least 50 cc. of serum must be had for the determination of diaminophosphatides in order to obtain a sufficient amount of Reineckate which can be weighed with enough accuracy.

Preparation of Dry Serum—50 cc. of serum are dried in a flat, glass dish as described above for stromata. The last traces of moisture are removed by placing the material in a vacuum desiccator. The dried serum is removed quantitatively from the dish. It is very difficult to scrape the brittle substance without losing some from the dish. Therefore, the dish is covered with a sheet of paper to prevent losing particles during the scraping. It is necessary to transfer the total amount of dry serum quantitatively in the thimble because the results in the case of serum are

TABLE I
Analysis of Stroma

Diagnosis	Dry material	Total extraction residue	Free cholesterol	Total lipid P	Total phospholipid	Diaminophosphatide	Monoaminophosphatide (total phospholipid - diamino)	Ratio, diamino to mono- amino
	gm.	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	
Normal adult.....	0.688	24.5	5.82	0.672	16.8	4.06	12.74	1:3.14
“ “	1.237	24.1	6.00			5.25		
“ “	1.150	29.8	5.74	0.625	15.6	5.82	9.78	1:1.68
“ “	2.3035	26.9	5.03	0.385	9.63	5.00	4.63	1:0.93
Diabetes mellitus.....	1.315	24.6	5.55	0.586	14.65	5.17	9.48	1:1.83
Essential hypertension ...	2.430	23.8	5.40	0.477	11.90	4.35	7.55	1:1.74
Cardiac failure.....	2.190	28.2	5.73			4.18		
Polycythemia.....	1.3217	23.5	5.60	0.506	12.65	4.24	8.41	1:1.98
Diabetes.....	1.550	21.5	5.27	0.483	12.1	4.88	7.22	1:1.48

calculated from the applied volume and not from weight as is the case with stromata. The serum is ground to a fine powder, mixed with sea sand, and extracted as described above for stromata. Aliquot parts of the extract made up to 50 cc. are taken for analysis as described in the general part of the paper.

The normal figures for diaminophosphatides in serum (Table II) according to our gravimetric method are about 100 to 180 mg. per cent. Normal values for total phospholipids are 200 to 350 mg. per cent. If the difference of total phospholipids to the amount of diaminophosphatides is evaluated as monoaminophos-

phatides, we calculate 100 to 170 mg. per cent present as monoaminophosphatides (lecithin and cephalin) in serum. In comparing the values obtained from stromata and serum, we must consider that one is obtained by calculating from 100 mg. of dry weight of stromata and the other is based on the analysis of 100

TABLE II
Analysis of Serum

Experiment No.	Diagnosis	Amount	Total lipid P	Total phospholipid	Diaminophosphatides (sphingomyelins)	Monoaminophosphatide (total phospholipid - diamino)	Ratio, diamino to mono-amino
		cc.	mg. per cent	mg. per cent	mg. per cent	mg. per cent	
1	Normal adult	50	10.60	265	184	81	1:0.44
2	" "	95	6.15	154	86	68	1:0.79
3	" "	50	12.90	322	220	102	1:0.46
4	" "	50	11.20	280	115	165	1:1.43
5	" "	50	7.75	194	103	91	1:0.89
6	" "	50	11.88	297	119	178	1:1.50
7	" "	50	9.60	240	124	116	1:0.94
8	" "	50	8.80	220	115	105	1:0.91
9	" "	50	9.20	230	117	113	1:0.97
10	" "	50	9.40	235	93	142	1:1.53
11	Dog, normal	43	14.40	361	260	101	1:0.39

The values of diaminophosphatides are obtained by multiplying the weight of Reineckate by the factor 0.877, calculated only for polydiaminophosphatides. We are investigating at present the acetone-soluble part of this Reineckate which has been suggested above to be a derivative of a monomeric sphingomyelin. If this assumption should be proved, the above figures for polydiaminophosphatides will be divided into polymeric and monomeric diaminophosphatides (sphingomyelins) by multiplying for the appropriate factors.

cc. of fluid; thus the large difference in actual figures in stromata and serum is to be comprehended. However, when the ratio of diaminophosphatides to monoaminophosphatides in serum is calculated, it is elucidated that this ratio is very close to the same ratio in stromata, namely 1:1 to 1:2, and, respectively, that the ratio of diaminophosphatides to total phospholipids is 1:2 to 1:3.

The maxima of these ratios may be proved later, by additional experience, as a little too high. At present we are applying this method clinically to the study of metabolic changes.

SUMMARY

1. The Reineckate of diaminophosphatide is used for quantitative determination of diaminophosphatide. The general application of the method is described.

2. Normal figures for the content of diaminophosphatide in serum and stromata of red blood cells are reported.

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VITAMIN C STUDIES IN THE RAT AND GUINEA PIG*

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The important rôle played by the small intestine of the rat in the metabolism of incomplete diets as related to the vitamin C content of the liver and gut has been adequately discussed (1).¹ More definite evidence with respect to various diets and fatty liver degeneration induced by carbon tetrachloride inhalation indicated that the gut was the probable site of synthesis of ascorbic acid (2). A more detailed study of this problem was undertaken with the feeding of substances, inorganic as well as organic, which interfered with the normal metabolic activities of the liver and gut.

In addition to the above study with rats, the relationship between cortin and vitamin C was investigated in view of the reported beneficial results of a high sodium, low potassium diet in the case of adrenalectomized dogs (3). With such a salt diet, adrenalectomized dogs can be maintained in normal condition without cortin for a long period of time. Experimental details from several laboratories indicated that cortin was without any influence in delaying the onset of scurvy in guinea pigs. It was hoped that with a high sodium salt diet further information might be obtained to clarify the connection between cortin and vitamin C, since guinea pigs dying with severe scurvy show many symptoms at necropsy which are characteristic of dogs succumbing to adrenal deficiency.

* Much of the material upon which this paper is based was presented before the meeting of the American Chemical Society at Pittsburgh, September, 1936.

¹ A recent communication by Zilva (Zilva, S. S., *Biochem. J.*, **30**, 857 (1936)) criticizes to a certain extent the interpretation of the data presented in this publication.

EXPERIMENTAL

Young white rats, selected from the various litters, were placed at weaning age on the basal ration of Dog Chow (Ralston Purina Dog Chow). At the beginning of the test period various substances were intimately mixed with ground Dog Chow and fed to the rats. Fresh food was given daily (an intake of 10 gm. of diet per rat). In order to prevent loss by spilling, the diet was moistened with water. The weight of the substances given to the animals varied within fairly wide limits but, in general, it was the maximum amount that could be tolerated by the rat as noted by the consumption of the diet. Water was kept before the animals at all times and weekly body weights were recorded.

At the end of the test period the rats were killed by stunning, 18 to 20 hours after the last feeding. The weighing of the organs, extraction with 4 per cent trichloroacetic acid, titration with standardized 2,6-dibromophenol indophenol blue, and estimation of the ascorbic acid content of the organs were carried out as previously described (2).

The experimental details as regards scurvy in the guinea pig will be discussed in a later section.

Effect of Metallic Salts—The influence of metallic salts on the body organism has been adequately discussed and an extensive bibliography has been compiled (4, 5), so that no attempt will be made to list the numerous references to the literature on this subject. In the present study the dosage fed, as well as the duration of the test period, was sufficient to insure the full physiological response of the rats to the metallic salts. There was no difficulty on refusal of the rats to eat the diet, but a retardation in growth (body weight) was noted in many instances.

The data in Table I indicate that the vitamin C content of the liver and gut was not decreased markedly by any of the salts fed. Occasionally, the general tendency, which had been previously noted with various diets (1, 2), of the vitamin C content of the gut to be higher than that of the liver, was not observed. Such deviations from the general trend are not of any great significance if the possible catalytic oxidation of vitamin C by traces of metallic salts passing through the intestine is taken into consideration.

The absorption of inorganic salts is quite low, but sufficient

Effect of Metallic Salts on Ascorbic Acid Content of Organs

Average dosage metallic salts mixed with Dog Chow fed daily	mg.*	No. of animals and sex	Average weight		Test period	Final age when killed	Vitamin C content								Comparison of relative weights of liver and gut			
			Initial	Final			Adrenals	Liver	Small intestine	Spleen	Adrenals	Liver	Small intestine	Spleen	Liver	Small intestine	Liver	Small intestine
			gm.	gm.	days	days	mg. per organ	mg. per organ	mg. per organ	mg. per organ	mg. per organ	mg. per gm.	mg. per gm.	mg. per gm.	mg. per gm.	gm. per 100 gm. final body weight	gm. per 100 gm. final body weight	
25 CuSO ₄		5 M.	108	171	25	67	0.078	1.830	1.980	0.11	4.30	0.25	0.33	0.18	7.33	5.98	4.28	3.49
50 "		4 "	98	142	16	65	0.094	1.890	1.610	0.06	4.33	0.29	0.26	0.16	6.45	6.35	4.54	4.49
500 BeCO ₃		4 "	84	226	34	71	0.090	2.112	2.090	0.08	4.30	0.24	0.30	0.16	8.82	6.84	3.90	3.02
Controls.....		3 "	80	219	34	71	0.083	2.310	1.960	0.10	4.70	0.25	0.31	0.20	9.22	6.26	4.21	2.85
5 Pb acetate + 3H ₂ O.....		5 F.	124	160	16	70	0.137	0.995	0.955	0.06	4.25	0.16	0.19	0.15	6.14	5.03	3.83	3.14
5 As ₂ O ₃		5 "	130	152	16	70	0.106	1.113	1.318	0.05	4.18	0.15	0.21	0.14	7.23	6.14	4.74	4.03
5 HgCl ₂		4 "	110	136	23	70	0.098	0.821	1.125	0.05	3.96	0.16	0.22	0.14	5.06	5.08	3.72	3.73
Controls.....		3 "	125	164	17	70	0.116	0.895	1.083	0.06	4.20	0.14	0.20	0.14	6.48	5.40	3.95	3.29
14 3CdSO ₄ + 8H ₂ O.....		5 "	83	108	29	70	0.074	0.882	1.464	0.06	3.76	0.18	0.21	0.21	4.85	7.14	4.49	6.61
19 Co(NO ₃) + 6H ₂ O.....		5 "	87	127	29	70	0.090	0.980	0.950	0.06	3.79	0.21	0.22	0.15	4.69	4.31	3.70	3.40
Controls.....		3 "	82	159	29	70	0.112	0.888	1.070	0.06	3.61	0.14	0.20	0.16	6.08	5.44	3.82	3.42
228 MnCl ₂ + 4H ₂ O.....		4 "	104	146	17	60	0.080	0.888	1.206	0.05	2.78	0.16	0.23	0.18	6.07	5.35	4.15	3.66
1 uranyl NO ₃ †.....		4 M.	107	95	5	44	0.073	0.660	0.805	0.05	3.00	0.14	0.22	0.22	4.50	3.63	4.73	3.82
Controls.....		2 "	101	126	5	44	0.052	1.350	1.610	0.07	3.65	0.21	0.28	0.19	6.00	5.32	4.76	4.22
28 Th(NO ₃) ₂ †.....		6 "	146		4 hrs.	50	0.020	1.838	1.791	0.10	1.19	0.25	0.34	0.23	6.90	5.07	4.72	3.47
31 uranyl NO ₃ †.....		5 "	149		4 "	50	0.034	1.812	1.788	0.07	1.89	0.26	0.32	0.24	6.91	5.43	4.63	3.63
Controls.....		4 "	149			50	0.044	1.655	1.840	0.10	2.75	0.24	0.32	0.19	6.84	5.66	4.58	3.79

* The mg. values are approximate.

† Injected subcutaneously.

amounts may be stored in the tissues owing to a cumulative storage from large doses. The relation of ascorbic acid content of the organs to the amount of CuSO_4 fed to the rats is of biologic importance. *In vitro* experiments have indicated that aqueous extracts of liver inhibit the oxidation of ascorbic acid in distilled water even when Cu or Fe salts have been added (6). The doses of CuSO_4 given *in vivo*, as indicated in Table I, suggest that the body has a protective mechanism whereby the amount of vitamin C is maintained at a fairly high level in the organs. There is some indication that cysteine, cystine, and glutathione play a part in this protective mechanism (7).

The ability of the gut to increase in weight with the feeding of some salts and to approach or even exceed the weight of the liver presents many interesting problems in physiology. Ordinarily, in young immature rats the gut exceeds the liver in weight, but the reverse weight relationship is found in older rats and the vitamin C content of the gut and liver tends to decrease as the age of the rat increases (2). Injury of the intestinal epithelium by poisoning with metallic salts as sodium fluoride, copper sulfate, or cadmium sulfate may account for the marked increase in weight of the gut. The surprisingly normal values for vitamin C content of the liver and gut of rats fed certain salts, if compared with the relative changes in weight of these organs, indicate that the gut plays a prominent rôle in the synthesis of the antiscorbutic vitamin.

Appreciable amounts of ascorbic acid were present in the organs of rats poisoned with subcutaneous injections of uranyl nitrate (1 mg. daily for 5 days). Refusal of the animals to eat the diet satisfactorily during the last 3 days may account for the slight decrease in ascorbic acid content of the liver and gut. Injections of large doses of thallium or uranyl nitrate in the brief period of 4 hours decreased the vitamin C content of the adrenals only. The marked decrease in ascorbic acid content of the adrenals conforms with previously reported work which showed that thallium salts change the histologic structure of the adrenal cortex (8).

Effect of Organic Substances—Foreign organic substances introduced into the animal body by way of the gastrointestinal tract, injection, or other means are not usually considered in the nature of foodstuffs but as toxic substances. Such toxic substances may

either be converted into more active pathogenic substances (1,2-benzanthracene, producing cancer under certain conditions in some species of animals) or be detoxicated by conversion into some harmless compound which is eliminated for the greater part from the body through the urine. The gut as well as the liver is normally endowed with important detoxicating powers.

The results of feeding of organic substances at levels indicated in Table II conform in general with previous data which indicate that the concentration of ascorbic acid in the gut tends to be higher than that of the liver. The actual amounts of vitamin C in the organs, the relative increase in weight of the liver and gut (such an increase in weight may be taken as a measure of the detoxicating power of that organ), and the retardation in body weight depended to a large extent on the substances fed. It has been shown in other laboratories that many of these substances interfere in one way or another with the metabolic processes of the liver and gut.

Our present knowledge of the metabolism of aromatic compounds with the exception of benzene and naphthalene is very scanty. Naphthalenemercapturic acid and phenanthreneglycuronic acid have been isolated from the urine of rabbits fed the corresponding hydrocarbons respectively. Dihydroxydihydroanthracene (which may possibly be formed in the gut by intestinal bacteria), a glycuronic acid, as well as a mercapturic acid derivative of anthracene, has been isolated from the urine of anthracene-fed rats (9). The formation of such compounds as dihydroxydihydroanthracene (which rapidly reduces KMnO_4 in acetone) among other possible derivatives may account for the higher reducing capacity (which can scarcely be attributed to vitamin C) of the liver and gut of rats fed anthracene. Such an explanation may possibly hold true in the case of the phenanthrene-fed rats which also show a higher reducing capacity in the gut than could be normally expected and especially since the feeding of other ring compounds as picric acid, naphthalene, or β -naphthol showed no such increase in the reducing capacity in these two organs. These results indicate that considerable caution should be used in the interpretation of data obtained by dye methods. Many substances, under certain conditions, may reduce the indophenol indicator without being antiscorbutic in nature. The organic

TABLE II
Effect of Organic Substances on Ascorbic Acid Content of Organs

Substances intimately mixed with Dog Chow fed daily	No. of animals and sex	Average weight		Test period	Final age when killed	Vitamin C content								Comparison of relative weights of liver and gut			
		Initial	Final			Adrenals	Liver	Small intestine	Spleen	Adrenals	Liver	Small intestine	Spleen	Liver	Small intestine	Liver	Small intestine
				gm.	gm.												
200 mg. * cholesterol.....	4 F.	94	151	21	58	0.095	1.125	1.050	0.07	3.80	0.15	0.22	0.19	7.33	4.86	4.85	3.21
15 " picric acid.....	4 "	91	105	22	58	0.060	0.740	0.976	0.10	3.34	0.14	0.21	0.15	28.4	5.53	5.02	4.31
Controls.....	3 "	85	141	22	59	0.078	0.843	1.081	0.05	3.50	0.12	0.21	0.16	6.71	5.20	4.76	3.68
100 mg. naphthalene.....	4 "	95	112	17	60	0.048	0.703	0.770	0.04	2.56	0.13	0.16	0.17	5.64	4.89	5.03	4.36
400 " anthracene.....	4 "	99	130	17	60	0.098	1.476	1.350	0.08	4.08	0.20	0.27	0.17	7.39	5.05	5.68	3.88
100 " phenanthrene†.....	4 "	99	107	17	60	0.070	1.275	1.185	0.06	4.04	0.16	0.26	0.22	7.75	4.65	7.24	4.34
20 " oxalic acid.....	4 "	96	143	17	60	0.112	0.900	1.008	0.08	3.82	0.15	0.20	0.14	6.19	5.03	4.30	3.51
Controls.....	4 "	95	141	17	60	0.109	0.939	1.004	0.08	3.99	0.15	0.19	0.23	6.52	5.14	4.62	3.62
0.10 cc. bromobenzene.....	5 "	75	83	36	72	0.040	0.392	0.525	0.03	2.38	0.07	0.13	0.10	5.66	4.02	6.83	4.85
0.10 " "	4 M.	118	145	22	65	0.067	0.930	1.050	0.06	3.87	0.11	0.20	0.21	8.69	5.19	6.00	3.58
0.05 " nitrobenzene.....	4 "	117	121	22	65	0.072	1.160	1.385	0.59	3.47	0.19	0.30	0.31	6.21	4.67	5.13	3.85
0.30 " benzene.....	3 "	120	185	22	65	0.088	1.680	1.760	0.03	3.98	0.23	0.28	0.14	7.44	6.29	4.00	3.40
23 mg. iodoacetic acid†.....	4 "	106	158	22	65	0.065	1.170	1.565	0.06	3.35	0.17	0.19	0.16	6.88	8.20	4.34	5.19
Controls.....	3 "	117	215	22	65	0.072	2.160	1.780	0.12	3.43	0.26	0.27	0.24	8.57	6.63	4.00	3.08
100 mg. β-naphthol.....	4 "	115	158	14	55	0.035	1.888	1.787	0.13	2.17	0.23	0.29	0.20	8.18	6.08	5.18	3.84
Controls.....	2 "	123	203	14	55	0.041	2.466	2.466	0.10	2.25	0.26	0.36	0.23	9.35	6.68	4.60	3.29

* The mg. values are approximate.

† Higher levels of phenanthrene were not eaten.

‡ Average dose, 40 mg. given the last 9 days of the test.

substances fed to the rats did not reduce the dye (the substances being dissolved in alcohol), but it is possible that intermediate compounds formed in the body from such substances may have the property of reducing the indophenol indicator.

The addition of iodoacetic acid to the diet yielded data which are of significance in relation to previously reported findings on the function of the intestinal mucosa in the absorption of carbohydrates and fats. The synthesis of phospholipids is checked by both phlorhizin and iodoacetic acid, but the absorption of fats is only slightly diminished by the former agent and completely inhibited by the latter substance (10). The phosphorylation mechanism of glucose, fructose, and galactose in the intestinal mucosa, as well as the rate of absorption of these sugars, is interfered with by iodoacetic acid (11). Halogenacetic acids have been shown to react with sulfhydryl compounds, chiefly glutathione and cysteine, and this action provided an adequate explanation for the destruction of glyoxalase activity by such acids (12). Ascorbic acid has been shown to reduce the inhibitory action of iodoacetic acid on the production of acetoacetic acid from butyric or crotonic acid on liver slices (13).

A marked difference exists in the response of the rat to phlorhizin and iodoacetic acid—the vitamin C concentration in the liver and gut being unaffected by phlorhizin (2), and decreased, as indicated in Table II, by iodoacetic acid. *In vitro* experiments indicated that iodoacetic acid did not reduce the amount of dye required for titration of ascorbic acid (0.05 mg. of ascorbic acid plus 8 mg. of iodoacetic acid required the same amount of indicator as ascorbic acid alone). Incubation experiments have likewise indicated that the protective power of liver in relation to ascorbic acid oxidation was unaffected by iodoacetic acid (7). Consequently, the feeding of iodoacetic acid interferes in some way with the synthesis of vitamin C in the rat—probably by the inhibition of the functions of the intestinal mucosa as regards the metabolism and absorption of foodstuffs.

The feeding of the benzene derivatives also yielded data which are of interest as regards the synthesis of vitamin C in the rat. Bromobenzene conformed with iodoacetic acid in the decrease of ascorbic acid in the liver and gut; nitrobenzene showed a decrease in the liver only while benzene was without any apparent effect.

The significant amounts of vitamin C in the organs of rats fed nitrobenzene are of interest if the physiological action of this substance is taken into consideration—methemoglobin formation in the blood and the markedly enlarged spleen. Considerable difficulty was encountered in the determination of a satisfactory end-point of the spleen extracts of the nitrobenzene-fed rats so that the values for the vitamin C content in that organ are higher than that actually present.

Bromobenzene leads to fatty degeneration of the liver but the parallel decrease of ascorbic acid in the liver and gut of the rats fed this substance as well as those fed iodoacetic acid suggests that there is a direct interference (the exact mechanism of which is unknown at present) with the synthesis of vitamin C. The decrease in ascorbic acid capacity of the liver and gut by such halogenated compounds can scarcely be attributed to vitamin C playing a major rôle in the detoxication of these substances. Such a decrease can be more readily explained if it is assumed that the gut is the probable site of synthesis of vitamin C which is then absorbed and utilized in the liver. Any interference with the functions of the intestinal mucosa by such halogenated compounds then would lead to a decrease in the vitamin C content in both of these organs.

Relationship of High Sodium Salt to Scurvy—The details as to selection of animals, diet, and scoring of scorbutic symptoms, described in the Sherman-Smith monograph (14), were followed. The animals were divided into two groups, one which received the usual basal diet plus tap water to drink, and the other which received the high salt diet (55 gm. of a 2:1 mixture of NaCl and sodium citrate per kilo of diet) plus a 0.9 per cent solution of these salts to drink. No difficulty was encountered in the refusal of the guinea pigs to consume the salt ration. On this salt diet about 4 to 5 times as much total salt is consumed as with the usual basal diet. The amount of salt mixture consumed was on a higher level than that fed to adrenalectomized dogs (approximately 1 gm. per kilo (3) in comparison to 2 to 3 gm. per kilo for the guinea pigs). To maintain the adrenalectomized dogs in normal condition without cortin, sodium citrate or sodium bicarbonate must be given in conjunction with sodium chloride. At the end of the test period the guinea pigs were killed by stunning, scored for scorbutic

TABLE III
Comparison of Diets in Scurvy

Diet	Vitamin C per os daily	No. of animals and sex	Average weight of diet eaten daily	Average weight		Average scurvy score (highest possible 24)	Average survival days	Vitamin C content*								Weight of adrenals mg. per 100 gm. final body weight
				Initial	Final			Adrenals	Liver	Small intestine	Spleen	Adrenals	Liver	Small intestine	Spleen	
Sherman-Smith basal	0	4 F.		357	219	19	19	0.014	0.33	0.15	0.019	0.03	0.02	0.01	0.01	177
	0	1 M.†		512	260	22	40									188
	0.25	2 F.		440	462	9	73	0.019	0.37	0.22	0.019	0.05	0.01	0.02	0.01	94
	0.25	1 M.		376	620	Trace	73	0.026	0.40	0.30	0.019	0.05	0.02	0.02	0.02	67
High sodium salt	0.50	2 "		328	622	0	73									82
	0	5 "	17	362	256	18	23									151
	0	1 "†	13	494	280	21	23	0.013	0.31	0.21	0.013	0.04	0.01	0.02	0.01	146
	0.25	3 "	26	344	523	8	72	0.014	0.36	0.20	0.014	0.04	0.01	0.02	0.02	77
	0.25	2 "	26	355	625	Trace	72	0.021	0.38	0.27	0.019	0.05	0.01	0.02	0.02	81
	0.50	2 "	30	312	615	0	72									

* Guinea pigs killed by stunning.

† Had been fed 0.5 mg. of vitamin C for 30 days previously.

symptoms, the organs extracted with trichloroacetic acid, titrated with standardized 2,6-dibromophenol indophenol blue, and estimated for vitamin C content.

The data in Table III indicate that there was no significant difference in survival period or scorbutic symptoms of the guinea pigs on the basal diets alone. Subnormal (0.25 mg.) as well as normal (0.5 mg.) doses of ascorbic acid likewise showed no marked deviations in response of the guinea pigs on the diets. A slight individual weight variation was apparent, but this weight variation is often encountered in vitamin C assay work and may be more pronounced as regards sexes of the experimental animals. The titration values of the organs, survival period, scoring of scorbutic symptoms, etc., conform with previously reported results (15), that in the guinea pig vitamin C is the essential factor in preventing scurvy and that cortin is without any effect in delaying the onset of scurvy. Adrenalectomized dogs can be maintained in normal condition without cortin on a high sodium salt diet because they synthesize their own vitamin C. Guinea pigs dying of scurvy show a much more marked hypertrophy of the adrenals (based for uniformity on weight of adrenals in mg. to 100 gm. of final body weight) than those which had received subnormal or normal doses of ascorbic acid.

SUMMARY

1. The feeding of metallic salts, some of which poison the intestinal epithelium, does not prevent the synthesis of vitamin C in the rat. The body has a protective mechanism which prevents the catalytic oxidation of vitamin C with CuSO_4 .

2. Halogenated compounds, as bromobenzene or iodoacetic acid, interfere with the synthesis of vitamin C in the rat. Other organic substances fed did not show this property.

3. Guinea pigs on a high sodium salt diet (a mixture of sodium chloride and sodium citrate added to the usual basal diet) succumb in the usual time interval and with the same severity of scorbutic symptoms as on the Sherman-Smith basal diet. Vitamin C is the limiting factor in preventing scurvy and, in those animals which are susceptible to this deficiency disease, is necessary for the utilization of the cortical hormone or the salt mechanism apparently controlled by cortin.

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THE DETERMINATION OF ZINC IN BIOLOGICAL MATERIAL

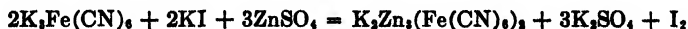
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Several investigators have already reported on the occurrence of zinc in the pancreas, Lutz (1), Delezenne (2), and recently Fisher and Scott (3). In the estimation of zinc in biological material, ashing was employed. This is a tedious procedure and there is always the possibility of the volatilization of zinc due to overheating. Moreover, since zinc along with other metals such as magnesium, copper, iron, cobalt, nickel, etc., is present in small amounts in the tissues, its separation renders the gravimetric procedure quite hazardous. Of course, the quantitative estimation of zinc by spectrographic analysis can be performed fairly accurately, but such apparatus is not always available and it requires an experienced operator.

In 1929 and later in 1933, Lang (4) published a method for the estimation of zinc in aqueous solutions by treating the slightly acidified solution of zinc with an excess of potassium ferricyanide and potassium iodide, whereby iodine is liberated and titrated against a standard solution of thiosulfate. Lang found that 1 cc. of 0.1 N sodium thiosulfate is equivalent to 10 mg. of zinc. Hibbard (5) confirmed Lang's findings and adopted the method for the estimation of zinc in soil and plants. The reaction involved was expressed as follows:



In this investigation we made use of the principle involved in the above equation for the estimation of zinc in proteins and in tissues, and determined the zinc content of the pancreas of various species.

Reagents—

0.5 per cent starch.

10 per cent potassium iodide. A fresh solution is prepared daily.

0.001 N sodium thiosulfate. This is prepared daily from a stock solution of 0.1 N sodium thiosulfate, and standardized against a standard solution of zinc containing 0.1 mg. per 1 cc.

Zinc standard. 1 gm. of pure zinc is dissolved in concentrated hydrochloric acid and diluted to 1 liter. 1 cc. contains 1 mg. of zinc and requires 10 cc. of 0.001 N sodium thiosulfate.

Potassium ferricyanide. A fresh 1 per cent solution is prepared daily.

Special phosphate. To 50 cc. of c.p. orthophosphoric acid (H_3PO_4) sufficient 10 per cent potassium hydroxide is added to bring the reaction to about pH 3.0. This is diluted to 1 liter.

Special ammonium acetate. Approximately 2 M ammonium acetate buffer, with a pH of about 6.0, is prepared.

Method

250 gm. of ground fresh pancreas are treated with 3 volumes of 4 per cent trichloroacetic acid, thoroughly stirred for at least 1 hour, allowed to stand overnight, and filtered through a hardened filter paper. The aqueous trichloroacetic acid filtrate contains all the zinc in solution. Samples (such as 200 cc. or as may be desired) of this filtrate are transferred to 250 cc. capacity centrifuge bottles; concentrated ammonium hydroxide is added until the reaction is alkaline to litmus, whereupon 5 cc. of the special ammonium acetate buffer are added, stirred, and followed by 1 or 2 cc. of concentrated acetic acid. The mixture is saturated with hydrogen sulfide, allowed to stand overnight, and centrifuged. The precipitate is next dissolved in about 1 or 2 cc. of hot 5 N hydrochloric acid and the solution is quantitatively transferred to a 50 or 100 cc. Pyrex flask. The liquid is evaporated to dryness over an asbestos-covered hot-plate until the excess of hydrochloric acid is almost completely driven off. While the flask is still warm, 5 cc. of 0.02 N sulfuric acid are slowly introduced along the sides of the flask, followed by 5 to 10 cc. of distilled water and 1 cc. of the special phosphate reagent. The liquid is boiled for 1 or 2 minutes, removed from the hot-plate, and allowed to cool to room temperature. It is now ready for titration.

Titration of Zinc—1 cc. of 0.5 per cent starch and 1 cc. of 10 per cent potassium iodide are added. The mixture should, at this stage, be colorless. However, if after 1 minute iodine is liberated owing to traces of copper or some other ions, 0.001 N sodium thiosulfate is added 1 drop at a time until the solution is clear. Then 1 cc. of 1 per cent potassium ferricyanide is introduced. The mixture is stirred and allowed to stand for 1 minute. The iodine liberated is titrated against the standard 0.001 N sodium thiosulfate until the addition of 1 drop of this reagent does not cause any change in the yellowish color at the point of contact with the liquid. 1 cc. of 0.001 N sodium thiosulfate is equal to 0.1 mg. of zinc.

EXPERIMENTAL

Experiment 1—To a known amount of zinc in solution (in 15 cc. centrifuge tubes) sufficient trichloroacetic acid is added to bring about a concentration of 3 to 4 per cent. The acidity is neutralized with 5 N ammonium hydroxide and 1 cc. of the special ammonium acetate is added, followed by 1 or 2 cc. of 2 N acetic acid.¹ The mixture is saturated with hydrogen sulfide and the tubes allowed to stand overnight and then centrifuged. The clear supernatant liquid is decanted and the precipitate is dissolved in 0.5 cc. of 5 N hydrochloric acid. The contents of the tubes are transferred quantitatively (with hot distilled water) into a 50 cc. or 100 cc. Pyrex flask. The liquid is carefully evaporated to dryness over an asbestos-covered hot-plate; 5 cc. of 0.02 N sulfuric acid are introduced, along with 1 cc. of special phosphate reagent, and the sides of the flask washed with distilled water. The liquid is allowed to boil for 1 or 2 minutes, removed from the hot-plate, and cooled to room temperature. Zinc is next titrated as described in the method. The results of this experiment are found in Table I.

Determination of Zinc in Presence of Iron

In the determination of zinc by the method described in this paper, the removal of iron is not essential, as it is in the gravimetric

¹ For the quantitative precipitation of zinc with hydrogen sulfide there are two prerequisites: (a) The solution must contain an excess of ammonium sulfide and (b) the acidity of the solution must be well controlled. For this purpose ammonium acetate and acetic acid are added.

method. It was observed in this laboratory that in solutions containing a mixture of zinc and iron in various proportions, even up to 5 mg. of iron to every 0.1 or 0.2 mg. of zinc (a ratio of 50 or 25 to 1) the addition of the acid phosphate reagent inhibits the reaction of iron with ferricyanide and potassium iodide. This is due to the formation of the insoluble iron phosphate in slightly acid solution. The zinc can then be quantitatively estimated in the manner described. It was further observed that when iron phosphates are absent, or present in moderate amounts, the zinc reaction proceeds fairly rapidly (within 1 minute) after the addi-

TABLE I

Estimation of Zinc Added to Solution of Approximately 4 Per Cent Trichloroacetic Acid, and to Concentrated Insulin A-8 in 4 Per Cent Trichloroacetic Acid

Experiment No.	Zinc		
	Amount added	Total amount found	Total amount found less zinc in sample
	mg.	mg.	mg.
1	0.10	0.10	
2	0.25	0.255	
3	0.32	0.31	
4	0.60	0.59	
5	1.04	1.09	
A-1 (Control)		0.68	
A-2	4.25	5.10	4.32
A-3	7.12	7.55	6.87
A-4	9.50	10.01	9.33

tion of the last reagent. However, in the presence of large amounts of iron phosphate, as in the proportions mentioned above, the liberation of iodine is definitely retarded, necessitating a 3 minute wait before titration.

Experiment 2—It was observed that by using the well known method of precipitating proteins with 3 to 4 per cent trichloroacetic acid, the zinc associated with the proteins remains in the filtrate. In order to determine whether or not the zinc is quantitatively recovered the following experiments were performed.

To each of three 100 mg. samples of dry insulin powder, 20 units per 1 mg., zinc was added and the samples were dissolved in dilute

hydrochloric acid. To each 10 cc. of insulin protein in solution 5 cc. of 10 per cent trichloroacetic acid were added. This quantity was found sufficient for the complete precipitation of the insulin protein. See Table I for the results.

A solution of crystalline insulin prepared in this laboratory, containing a known amount of zinc, was used for this demonstration. The zinc content of this material had been determined spectrographically and was shown to be in the neighborhood of 0.9 mg. per 1000 units. The zinc content of this crystalline

TABLE II

Estimation of Zinc in Samples of Crystalline Insulin, Lots 1657-I and 2499-I

Experiment No.	Technique	Material used		Zinc found	
		Lot No.		Per sample	Per 1000 units
			units	mg.	mg.
B-1	Ashing as sulfate	1657-I	1000	0.942	0.942
	" " "	1657-I	1000	0.932	0.932
B-2	" " "	2499-I	1050	0.950	0.905
	" " "	2499-I	1050	0.970	0.925
B-3	Trichloroacetic acid extraction	1657-I	1000	0.924	0.924
	" "	1657-I	1000	0.914	0.914
B-4	" "	2499-I	1050	0.913	0.870
	" "	2499-I	1050	0.915	0.870
B-5	Digestion	1657-I	1000	0.891	0.891
	"	1657-I	1000	0.900	0.900
B-6	"	2499-I	1050	0.883	0.840
	"	2499-I	1050	0.950	0.905

material was analyzed in duplicate by three different preliminary procedures. (1) 1000 units of crystalline insulin were brought to dryness and ashed as sulfates in platinum crucibles. The ash was dissolved in hydrochloric acid and zinc was estimated. (2) 1000 units of crystalline insulin in solution were precipitated with trichloroacetic acid, the filtrate was recovered quantitatively, and zinc was estimated. (3) 1000 unit samples of crystalline insulin in solution were digested with concentrated sulfuric acid, potassium sulfate, and nitric acid. The clear digested material was neutralized and the process of sulfiding was performed. The results of these experiments are shown in Table II.

TABLE III

Estimation of Zinc in Pancreatic Tissue with and without Added Zinc

Experiment No.	Zinc, per kilo		
	Amount added	Total amount found	Net amount
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
C-1	None	27.06	27.06
	"	26.20	26.20
C-2	20	47.00	27.00
	20	47.00	27.00

TABLE IV

*Amount of Zinc Found in Fresh Pancreas of Beef, Calf, Sheep, and Hog.
Extraction with Trichloroacetic Acid*

Determination No.	Material	Amount found per kilo fresh pancreas	Average per kilo
		<i>mg.</i>	<i>mg.</i>
B-1	Beef	32.4	
	"	34.8	33.6
B-2	"	43.2	
	"	40.6	41.9
B-3	"	41.4	
	"	42.0	41.7
C-1	Calf	34.9	
	"	33.3	34.1
C-2	"	38.8	
	"	43.2	41.0
C-3	"	36.2	
	"	36.6	36.4
S-1	Sheep	19.9	
	"	19.04	19.5
S-2	"	24.0	
	"	25.0	24.5
S-3	"	24.8	
	"	24.7	24.75
H-1	Hog	42.9	
	"	44.4	43.6
H-2	"	35.2	
	"	34.6	34.9
H-3	"	31.0	
	"	28.2	29.6

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500 gm. of well ground pancreas were divided into two equal parts. 250 gm. were treated with 3 volumes of 4 per cent trichloroacetic acid (final volume was 1 liter), thoroughly mixed, allowed to stand overnight, and filtered. The filtrate was labeled C-1. To the other 250 gm., 20 mg. of zinc in solution were added and the ingredients intimately mixed; 3 volumes of 4 per cent trichloroacetic acid were added (final volume 1 liter), mixed, allowed to stand overnight, and filtered. This filtrate was labeled C-2. Zinc determination was then performed on Filtrates C-1 and C-2 according to the method described. The results of this experiment are shown in Table III.

Zinc Content of Pancreas

Experiment 3—1 kilo of fresh pancreas of each of the following species was analyzed for zinc: beef, calf, sheep, and hog. The pancreas of each species was divided into three lots; each lot was ground separately, and 250 gm. samples of each were used. To 250 gm. of ground tissues enough 4 per cent trichloroacetic acid was added to make up a volume of 1 liter. The mixture was thoroughly stirred for 1 hour, treated with a few cc. of toluene, and allowed to stand overnight. The following day each lot was filtered separately and zinc was determined in accordance with the method outlined. The results are shown in Table IV.

Zinc Content of Commercial Insulin

Experiment 4—As a matter of interest samples of different preparations of insulin found on the market were secured and zinc was determined by trichloroacetic acid extraction according to the method described. The results are as follows:

Preparation	Insulin per sample	Zinc found per 1000 units
	units	mg.
A	800	0.043
B	800	0.068
C	400	0.094
D	800	0.050

SUMMARY

A method for the microestimation of zinc in biological materials is described.

Values for the zinc content of the pancreas of beef, calf, sheep, and hog are given. These values are somewhat higher than those reported by Fisher and Scott (3).

Commercial insulin of different sources was observed to contain small amounts of zinc. These values are not constant but will vary considerably, one way or another, from batch to batch.

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STUDIES ON BIOLOGICAL OXIDATIONS

VII. THE OXIDATION OF ASCORBIC ACID IN BIOLOGICAL FLUIDS

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In a previous paper (1) we have shown that ascorbic acid, dissolved in buffered solutions of pH values below 7, although not autoxidizable, is readily oxidized by atmospheric oxygen in the presence of such minute traces of copper as cannot be detected by Kolthoff's reagent (2). Nevertheless, in tissues and certain biological fluids ascorbic acid is present mostly in its reduced form (van Eekelen, Emmerie, Josephy, and Wolff (3), Bacharach, Cook, and Smith (4), Bessey and King (5), and Gabbe (6)), although copper is found there in measurable quantities. In these fluids there exist inhibitory mechanisms which protect ascorbic acid from oxidation. There are, moreover, other biological fluids, mostly of vegetable origin, which oxidize ascorbic acid at a measurable speed, fluids in which there has been reported the presence of ascorbic acid-oxidizing "enzymes" (Szent-Györgyi (7), Zilva (8), Tauber, Kleiner, and Mishkind (9)). The rate of oxidation of ascorbic acid in these two classes of biological fluids and the effect of specific inhibitors have been studied in an endeavor to understand the mechanism of the protective action against ascorbic acid oxidation in those fluids belonging to the first group, as well as to discover in those fluids belonging to the second group the catalysts which do oxidize ascorbic acid.

EXPERIMENTAL

In these experiments the same care taken previously (1) to avoid contamination of fluids by heavy metals was observed. The oxidation of ascorbic acid was determined, in Warburg modified

vessels attached to Barcroft manometers, by measuring the oxygen consumption at 37° in the case of fluids of animal origin, and at 25° in that of fluids of vegetable origin. The fluids were kept in the main vessel, the ascorbic acid solution in the side arm of the vessel. When temperature equilibrium was reached, the fluids were mixed and the rate of ascorbic acid oxidation was followed. The oxidation of ascorbic acid in serum (at pH 7.39), in blood, and in cerebrospinal fluid (pH 7.20) was performed in a gas mixture containing 95 per cent O₂ and 5 per cent CO₂. The thermobarometer contained the fluids with no ascorbic acid. The copper content of the fluids was determined by Fischer and Leopoldi's method (10). The pH values were determined by the glass electrode. The ascorbic acid and glutathione were obtained from Hoffmann-La Roche. The buffers were prepared free of iron and copper.

Oxidation of Ascorbic Acid in Biological Fluids Possessing an Inhibitory Power

De Caro and Giani (11) reported that animal tissues and tissue extracts protected ascorbic acid from oxidation and suggested that glutathione might act as the inhibitor of this oxidation. This suggestion found support recently in Mawson's (12) experiments. The non-physiological conditions of these experiments are obvious; the animal tissues were ground, sometimes treated with strong acids, and oxidation of ascorbic acid was determined in extracts of such tissues. In the experiments here reported the oxidation of added ascorbic acid was followed in normal biological fluids with and without added CuCl₂. Among the fluids of animal origin, the following were taken: blood serum, cerebrospinal fluid, urine, saliva, and gastric juice of man; whole blood of dog; aqueous vitreous humor of cattle; cow's milk; among those of vegetable origin: orange, tomato, and grapefruit juices. In Table I is given the rate of oxidation of ascorbic acid in these fluids. In the absence of added catalyst all these biological fluids protected to varied degrees the oxidation of added ascorbic acid, the protection being complete in the case of gastric juice, milk, and orange, tomato, and grapefruit juices. It is of interest to note the similarity in the rates of oxidation of ascorbic acid in blood serum and that in whole blood, indicating that the presence of red cells has no influence on this oxidation. In order to determine the protective power of

TABLE I
Oxidation of Ascorbic Acid in Biological Fluids Possessing Inhibitory Mechanisms
Amount of ascorbic acid added, 0.02 mm. Temperature, 37° in fluids of animal origin; 25° in fluids of vegetable origin.

Fluid	Concentration of Cu in fluid milli-atom per l.	Concentration of CuCl ₂ added milli-atom per l.	pH	Time for half oxidation min.
Blood serum (man).....	2.93×10^{-2}		7.42	294 (Calculated) 32.6 c.mm. O ₂ consumed in 60 min.
" ".....	2.52×10^{-2}		8.50	148.6
" ".....	2.52×10^{-2}	7.28×10^{-2}	8.50	115.6
" (whole) (dog).....	1.89×10^{-2}		7.20	216.8 (Calculated) 33.8 c.mm. O ₂ consumed in 60 min.
Cerebrospinal fluid (man).....	5.03×10^{-2}		7.33	1900 (Calculated) 7.7 c.mm. O ₂ consumed in 120 min.
" ".....	5.03×10^{-2}		8.26	149.5
" ".....	5.03×10^{-2}	7.28×10^{-2}	7.30	46.1
Urine (man).....	7.06×10^{-2}		5.42	787 (Calculated) 9.3 c.mm. O ₂ consumed in 60 min.
" ".....	7.06×10^{-2}	7.27×10^{-2}	5.42	31.8
Saliva ".....	?		7.38	30
Gastric juice (man).....	?		4.52	No oxidation in 120 min.
Aqueous vitreous humor (cattle).....	2.52×10^{-2}		8.41	125.4
" ".....	2.52×10^{-2}	7.28×10^{-2}	8.41	20.4
Milk (cow).....	2.35×10^{-2}		6.54	No oxidation in 60 min.
" ".....	2.35×10^{-2}	7.28×10^{-2}	6.54	No oxidation in 60 min.
" ".....	2.35×10^{-2}	7.28×10^{-1}	6.54	275.4 (Calculated) 31 c.mm. O ₂ consumed in 70 min.
Orange juice.....	1.26×10^{-2}		3.66	No oxidation in 60 min.
" ".....	1.26×10^{-2}	7.28×10^{-2}	3.66	340 (Calculated) 21.5 c.mm. O ₂ consumed in 60 min.
Tomato ".....	9.45×10^{-2}		4.50	No oxidation in 60 min.
" ".....	9.45×10^{-2}	7.28×10^{-2}	4.50	948 (Calculated) 10.3 c.mm. O ₂ consumed in 60 min.
Grapefruit juice.....	7.09×10^{-2}		3.18	No oxidation in 60 min.
" ".....	7.09×10^{-2}	7.28×10^{-2}	3.18	134.3 (Calculated) 53.6 c.mm. O ₂ consumed in 60 min.

these fluids against the oxidation of added ascorbic acid, the concentration of copper in these fluids was determined by Fischer and Leopoldi's method. The protective power was ascertained by dividing the half oxidation time, in minutes, in the biological fluid by that in a buffer solution of identical pH value and copper concentration, since these two factors have an important rôle in the rate of oxidation of ascorbic acid. Besides the normal protec-

TABLE II

Protective Power of Biological Fluids against Oxidation of Ascorbic Acid

Protective power = time for half oxidation of added ascorbic acid in the biological fluid (in minutes) divided by the time for half oxidation in a buffer of identical pH value and Cu concentration.

Fluid	Protective power	
	Normal	Against added CuCl_2 (7.28×10^{-3} mM per liter)
Blood serum (man).....	18.6	12.7
Whole blood (dog).....	15.5	
Cerebrospinal fluid (man).....	22.0	6.6
Urine (man).....	16.2	1.7
Saliva.....	39.6?*	2.7
Gastric juice (man).....	Complete	
Aqueous vitreous humor (cattle).....	7.3	2.0
Milk (cow).....	Complete	22.9
Orange juice.....	"	8.7
Tomato ".....	"	25.2
Grapefruit juice.....	"	2.6

* The amount of copper in saliva was not determined. To obtain the protective power it was assumed that the concentration of copper in saliva was similar to that in blood serum.

tive power, the power to protect ascorbic acid from oxidation by added CuCl_2 was also determined (Table II). Blood serum, milk, and tomato juice had by far the greatest protective power against added CuCl_2 ; urine, saliva, and grapefruit juice, the least.

Undoubtedly, the property of these fluids to protect ascorbic acid from oxidation is due, as suggested by Mawson (12), to varied inhibitory factors. Of these factors, the most important must be those concerned with the inhibition of the catalytic power of

copper, since copper is present in all biological fluids in a concentration sufficient to produce a speedy oxidation of ascorbic acid (Lindow, Elvehjem, and Peterson (13)). This catalytic action of copper can be diminished by the formation of un-ionized copper complexes such as those formed with proteins (Ettisch, Sachsse, and Beck (14)) and with a number of amino acids (Borsook and Thimann (15)). As an example of such inhibition, there is given in Fig. 1 the rate of oxidation of ascorbic acid by CuCl_2 (0.0002 mM) as catalyst at a pH value of 6.34 with and without ovalbumin (6 per cent) and glycine (0.1 M glycine was added, a concentration

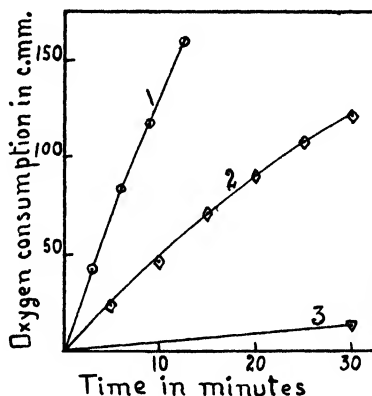


FIG. 1. The effect of proteins and amino acids on the oxidation of ascorbic acid by CuCl_2 (0.0002 mM). Amount of ascorbic acid, 0.02 mM ; pH, 6.34; temperature, 25°. Curve 1 represents phosphate buffer + CuCl_2 ; Curve 2, phosphate buffer + CuCl_2 + glycine (0.1 M per liter); Curve 3, phosphate buffer + CuCl_2 + ovalbumin (6 per cent).

which, according to Borsook and Thimann, is more than sufficient to keep the copper entirely un-ionized). The addition of ovalbumin to the phosphate buffer produced 92.5 per cent inhibition, and the addition of glycine, 64 per cent inhibition. An even more powerful inhibiting agent of the catalytic action of copper was found to be glutathione. This inhibiting effect of glutathione had already been observed by De Caro and Giani (11), Bersin, Köster, and Jusatz (16), and Mawson (12), but no satisfactory explanation was found. It was contended that the inhibition was due to the reducing power of glutathione, stronger than that of ascorbic acid.

The experiments reported in Table III seem to indicate that this inhibition is due rather to the strong affinity of glutathione for copper with the formation of a copper-glutathione complex already described by Hopkins (17). In these experiments, 0.01 mM of ascorbic acid was oxidized in phosphate buffer, pH 6.34, with CuCl_2 as catalyst, and the rate of oxidation was compared with that of identical solutions containing varying amounts of glutathione. Some experiments were performed at a pH value of 7.33 with similar results. As can be seen in Table III, it is not the ratio of ascorbic acid to glutathione which determines the inhibitory

TABLE III

Effect of Glutathione on Oxidation of Ascorbic Acid with CuCl_2 As Catalyst

Amount of ascorbic acid, 0.01 mM. pH, 6.34 (phosphate buffer). Temperature, 25°. Inhibition was calculated by comparing the rate of oxidation of ascorbic acid in phosphate buffer + CuCl_2 with the rate of oxidation in the same solution plus glutathione.

Amount of reduced glutathione	Amount of CuCl_2	Inhibition
mM	mM	per cent
0.01	0.0002	Complete
0.01	0.0001	"
0.005	0.00005	"
0.0005	0.00005	"
0.0002	0.00005	"
0.0001	0.00005	"
0.00005	0.00005	90
0.00001	0.00005	10
0.000001	0.00005	None

power of glutathione, but the ratio of CuCl_2 to glutathione. The inhibitory power was complete as long as there existed more than 1 molecule of glutathione per atom of copper. According to Hopkins the copper-glutathione complex contains 1 atom of copper per molecule of glutathione. When such a ratio was reached there was a slight oxidation of ascorbic acid (90 per cent inhibition); when the ratio of $\text{Cu}:\text{GSH}$ was 5:1, the inhibition was only 10 per cent, becoming nil when the ratio was 50:1. Since a ratio of $\text{Cu}:\text{GSH}$ of 1:2 is enough to produce complete inhibition of ascorbic acid oxidation by copper, a concentration of glutathione of

0.054 mm per liter (16.6 mg. per liter) would prevent entirely the oxidation of ascorbic acid in the blood, for the concentration of copper in the blood is about 0.027 milli-atom per liter. It would be possible to determine the ionization constant of the copper-glutathione complex by the degree of inhibition on the oxidation of ascorbic acid by ionic copper.

We may therefore conclude that glutathione, proteins, and amino acids (by the formation of un-ionized copper complexes) are the most important mechanisms for the protection of ascorbic acid from oxidation in those biological fluids possessing such inhibitory action.¹

*Oxidation of Ascorbic Acid by Biological Fluids Possessing
No Inhibitory Mechanisms*

In 1931 Szent-Györgyi (7) reported the presence in cabbage juice of an "enzyme" which oxidized ascorbic acid; Zilva (8) found the "enzyme" in apple juice; and Tauber, Kleiner, and Mishkind (9) found it in Hubbard squash juice. As ascorbic acid is readily oxidized by a number of reversible oxidation-reduction systems of suitable potential (such as copper, hemochromogens, a number of vegetable dyes), it could be predicted that the so called ascorbic acid oxidase would be found in a variety of biological fluids, namely among those having no inhibitory mechanisms. The reversibility of such oxidations, observed by Zilva, is no attribute of the "enzyme" but a property of ascorbic acid oxidized at the pH values of Zilva's experiments, as was shown previously in this laboratory (1).

As representative examples of this group of biological fluids there were selected vegetable juices extracted from apple, cabbage, yellow squash, peach, and watercress. Because of the low buffering power of these fluids, the ascorbic acid was previously neutralized with Na_2CO_3 so that the oxidations were performed without altering the normal hydrogen ion concentration of the biological fluid. As can be seen in Table IV, yellow squash juice oxidized ascorbic acid faster than all the other fluids examined, half oxidation being reached in 16.6 minutes. At this pH value and copper

¹ Eisler, Rosdahl, and Theorell (18) have just reported that the copper in serum is combined with serum albumin.

concentration, half oxidation of ascorbic acid in a phosphate buffer was reached in 30.8 minutes. The rate of oxidation corre-

TABLE IV

Oxidation of Ascorbic Acid in Biological Fluids Possessing No Inhibitory Mechanisms

Amount of ascorbic acid added, 0.02 mM. Temperature, 25°.

Fluid	pH	Concentration of Cu in fluid	Half oxidation time
		milli-atom per l.	min.
Apple juice.....	4.25	1.24×10^{-3}	43.9
Cabbage juice.....	5.10	9.44×10^{-3}	35.4
Yellow squash.....	5.94	8.03×10^{-3}	16.6
Lettuce juice.....	5.42	6.30×10^{-3}	22.3
Peach juice.....	3.76	1.73×10^{-3}	35.0
Watercress juice.....	4.62	6.73×10^{-3}	45.7

TABLE V

Effect of HCN and 8-Hydroxyquinoline on Oxidation of Ascorbic Acid in Biological Fluids Possessing No Inhibitory Mechanisms

Temperature, 25°.

Fluid	pH	Inhibition, per cent	
		HCN, 0.005 M per liter	8-Hydroxy- quinoline, about 0.002 M per liter
Phosphate buffer + CuCl ₂ , 0.0002 mM..	6.34	Complete	Complete
Buffer + 0.1 M glycine + CuCl ₂ , 0.0002 mM.....	6.34	"	"
Phosphate buffer + ferri-nicotine- hemochromogen.....	6.87	58.4	None
Apple juice.....		67.4	41.0
Cabbage juice.....		52.8	None
Yellow squash.....		92.4	"
Lettuce juice.....		58.4	43.9
Peach juice.....		61.4	42.8
Watercress juice.....		88.4	None

sponded neither to the pH value of the solution nor to the copper concentration.

In order to determine the catalysts responsible for the oxidation

of ascorbic acid in these fluids, two specific inhibitors were used (Table V): HCN, which inhibits oxidations produced by hemochromogens; and 8-hydroxyquinoline, which inhibits those produced by copper, the inhibiting effect of these substances having been previously established in buffer solutions containing CuCl_2 , copper-glycine, and ferri-nicotine-hemochromogen as catalysts. HCN at a concentration of 0.005 M per liter produced an inhibition

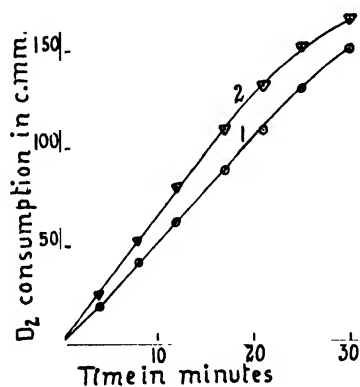


FIG. 2

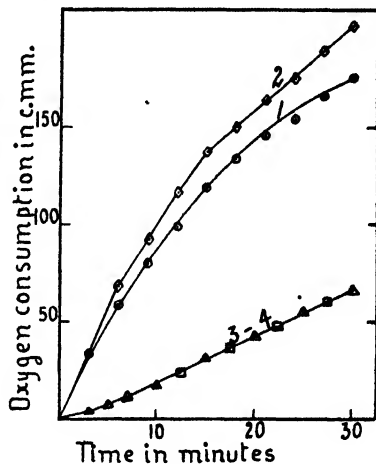


FIG. 3

FIG. 2. The oxidation of ascorbic acid by lettuce juice. Amount of ascorbic acid, 0.02 mM; pH, 5.42; temperature, 25°. Curve 1 represents lettuce juice; Curve 2, lettuce juice + CuCl_2 (0.0002 mM).

FIG. 3. The effect of glutathione on the oxidation of ascorbic acid by yellow squash and phosphate buffer plus ferri-nicotine-hemochromogen. Curve 1 represents ferri-nicotine-hemochromogen (hemin, 0.00015 mM; nicotine, 3.5 mM); Curve 2, ferri-nicotine-hemochromogen + glutathione (0.01 mM); Curve 3, yellow squash; Curve 4, yellow squash + glutathione (0.0005 mM). Amount of ascorbic acid, 0.02 mM.

of 58 per cent on the oxidation of ascorbic acid by the hemochromogen, while 8-hydroxyquinoline had no effect; the catalytic effect of copper was completely inhibited by both reagents. By the use of these inhibitors it was found that the oxidation produced by the juices of cabbage, yellow squash, and watercress was not due to copper catalysis, for the rate of oxidation was identical with and without 8-hydroxyquinoline. On the other hand, the oxida-

tion by the juices of apple, lettuce, and peach was found to be partly due to the copper present, as shown by the partial inhibition produced by 8-hydroxyquinoline. HCN (0.005 M) inhibited to a great extent (90 per cent) the oxidation produced by yellow squash, and only 50 per cent the oxidation by cabbage juice. The addition of CuCl_2 (0.0002 M) to cabbage juice did not increase appreciably the rate of oxidation (Fig. 2).

It has been shown previously that glutathione inhibits the oxidation of ascorbic acid with copper as catalyst. Since the inhibition depended on the ratio Cu:GSH, it was concluded that the effect was specific for copper catalysis. This conclusion finds further support in the experiments reported in Fig. 3, in which the effect of glutathione on the oxidation of ascorbic acid by yellow squash and by phosphate buffer containing ferri-nicotine-hemochromogen was studied. In both of them glutathione failed to inhibit the oxidation. In the case of yellow squash the rates with and without glutathione were identical (yellow squash does not oxidize glutathione); in the case of ferri-nicotine-hemochromogen, the rate of oxidation in the presence of glutathione was slightly greater, probably due to a slow oxidation of the glutathione added to the oxidation of ascorbic acid. It is therefore quite probable that hemochromogens are the main catalysts for the oxidation of ascorbic acid in these biological fluids possessing no inhibitory mechanisms.

SUMMARY

Biological fluids may be divided according to their behavior towards ascorbic acid into two groups: those possessing inhibitory mechanisms protecting the ascorbic acid from oxidation and those devoid of these mechanisms. Fluids of animal origin and some vegetable fluids (those containing appreciable quantities of ascorbic acid) belong to the first group. Ascorbic acid is protected from oxidation there by the action of glutathione, proteins, and amino acids, which inhibit copper catalysis. Fluids of vegetable origin (those containing very little ascorbic acid) belong to the second group. Ascorbic acid is oxidized in these fluids by a variety of oxidizing catalysts, copper and hemochromogens being probably the most common, as shown by the effect of inhibitors. The inhibiting effect of glutathione is specific for copper catalysis and

has no action on the rate of oxidation of ascorbic acid by ferri-nicotine-hemochromogen or by yellow squash juice.

Addendum—Quite recently, Hopkins and Morgan (19) have reported in an extremely interesting paper that when ascorbic acid and glutathione are added to cauliflower juice (containing ascorbic acid oxidase) in a ratio of 1:2, ascorbic acid is wholly protected from oxidation, while glutathione is oxidized at exactly the same rate as was ascorbic acid when alone. These findings, which would apparently contradict the experiments reported in Fig. 3, do not in reality contradict them, for in these experiments in which we studied the influence of glutathione on the oxidation of ascorbic acid by yellow squash juice, the ratio of ascorbic acid to glutathione was 1:0.025; i.e., the concentration of glutathione was too small to produce the effect found by Hopkins and Morgan.

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A PHOTOELECTRIC METHOD FOR RECORDING FAST CHEMICAL REACTIONS AND ITS APPLICATION TO THE STUDY OF CATALYST-SUBSTRATE COMPOUNDS*

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In the course of the decomposition of monoethyl hydrogen peroxide by liver catalase there is formed an intermediate compound with a characteristic absorption spectrum. The intermediate is unstable; it breaks down to form the free enzyme and product molecules (1). The rate of formation of the enzyme-substrate compound is great compared with that of the over-all reaction (2). A similar intermediate has been observed in the reaction between methemoglobin and ethyl hydrogen peroxide (1, 3). A quantitative study of the kinetics of the combination between catalyst and substrate was undertaken in view of its bearing on the problem of enzyme action and of catalysis in general. The principle of the flow method, as applied to the study of fast chemical changes by Hartridge and Roughton (4), Millikan (5), and other workers, proved to be impractical for the present purpose. The preparation of the required reactants in sufficient quantity and of sufficiently high concentration for extinction measurements in observation tubes presents one of the major difficulties. Furthermore, previous spectroscopic experiments indicating the existence of a lag period during the reaction between enzyme and substrate (2) made a continuous and automatic recording of the process preferable to the selection of a restricted number of observation points along the flow tube, representing the time abscissa, as was done by the Cambridge workers.¹

* This work was aided by a grant from the Elizabeth Thompson Science Fund, the assistance of which is hereby gratefully acknowledged.

¹ The modification of the flow method by Thiel and Logemann (6) permits a continuous registration over a short period, but the apparatus is rather complex and expensive in construction.

Consequently a method was developed to suit the particular needs of the present problem, although it is perhaps sufficiently flexible to find application in other problems of a related nature. The method is meant to supplement rather than to supplant the flow method.

Principle

Light of high intensity passes a monochromator. The monochromatic radiation, selected in such a manner as to correspond to the position of the absorption bands to be studied, penetrates a cell containing the reaction mixture and strikes the cathode of a photoelectric cell. The photoelectric current is amplified by a thermoionic valve circuit. The amplifier is connected with a string galvanometer equipped with a film camera. At the beginning of the experiment the substrate is added to the solution of the catalyst contained in the absorption cell. Rapid mixing free from appreciable mechanical and electrical disturbance is achieved by a special injector mechanism. The changes in light absorption accompanying the chemical reaction cause deflections of the galvanometer string, which are continuously recorded on the photographic film. By a grid and a time marker the film is provided with a coordinate net background. The abscissa permits direct reading of the time lapsed at any stage of the reaction. The unit of time is 0.04 second. By varying the speed of film transport the process of recording may be adapted to the rate of the reaction under study. The ordinate may be calibrated to correlate galvanometer deflections with concentrations of reactants. Proportionality between photoelectric current and light intensity can be tested for by a rotating sector which decreases the apparent light intensity by a known increment.

EXPERIMENTAL

Description of Apparatus

The arrangement of the elements required for the method is schematically shown in Fig. 1. The injector is fixed vertically above the absorption cell.

Optical Arrangement—As light sources, both for the monochromator and for the string galvanometer, No. 1183 automobile head light lamps (6 volts) are used. They are fed from ac-

cumulator batteries. The light of one lamp is concentrated on the front slit of the monochromator by means of three plane-convex lenses (see Fig. 1), the last of which is a cylindrical lens. As a monochromator an Adam Hilger wave-length spectrometer is used, the ocular of which is replaced by an asymmetrical slit. As absorption cells either 0.5 or 1.0 cm. deep cells with cemented end-plates and round middle part are used. The capacity of the cells is between 2 and 3 cc. In the earlier experiments a rectangular cuvette of 1.2 cm. depth and 15 cc. volume was used. The beam penetrating the absorption cell is projected on the cathode of the photoelectric cell in the form of a rectangle of

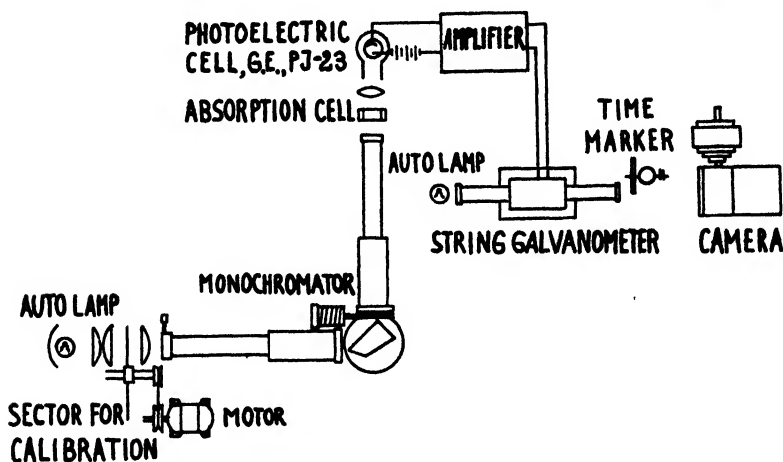


FIG. 1. Arrangement of apparatus.

about 0.5×2.0 cm. The cathode has the shape of a half cylinder, the concave side of which is turned towards the light. A thin film of cesium is deposited on the silver cathode. In earlier experiments an evacuated photocell (General Electric, type PJ-22) was tried. For the experiments described in this paper a gas-filled cell (General Electric, PJ-23) was employed. The camera recording the deflections of the string galvanometer is equipped with 6 cm. wide Eastman Kodak sensitized bromide paper film.

Electrical Circuit—The details of the electrical circuit are shown in Fig. 2.

The maximal anode voltage applied to the photocell is 90 volts. The range of deflection of the string galvanometer and consequently the sensitivity of the apparatus can be varied by a variable shunt of 6.000 ohms. Sometimes it has been found necessary to eliminate stray currents causing oscillations of the galvanometer string by means of a suitable filter containing a condenser of several microfarads. The photocell, the amplifier, and the string galvanometer form separate units which are properly shielded and earthed. An RCA-38 valve serves as the amplifier tube. The string galvanometer and the camera are components of an electrocardiograph apparatus.

Mixer Mechanism—The method of mixing stratified layers of the reactants by an electric stirrer (7), as applied to the direct

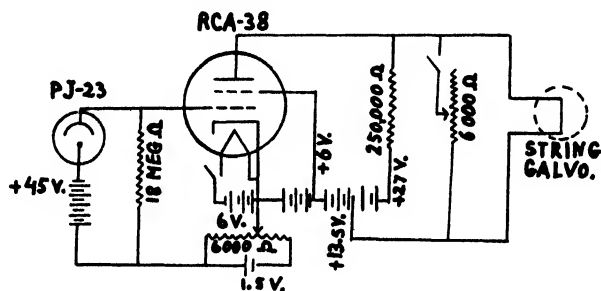


FIG. 2. Electrical circuit

spectroscopic study of the enzyme-substrate compound (2), proved to be unsatisfactory for the present purpose. The starting of the motor caused an induction current in the photocell-amplifier circuit. Furthermore, too much time is required for completion of the mixing.

In earlier qualitative experiments one of the authors had observed that simple injection of substrate solution into a stationary enzyme solution by means of a syringe seemed to bring about a rapid and complete mixing of the reactants, as far as direct visual observation was concerned. With suitable improvements this procedure has been found to be satisfactory for the present purpose.

In the final injector apparatus which is shown in Fig. 3, the fluid contained in a 2 cc. glass syringe is ejected by the release

of a long spring made from piano steel wire. The spring is under considerable tension even when the piston of the syringe is at its lowest point. The spring was made sufficiently long to render the distance of compression and expansion required for the discharge of fluid small compared with the total length. Thus the force exerted in the course of expansion may be considered to remain practically constant. The amount of solution to be discharged may be accurately limited by exchangeable spacing sleeves as shown in Fig. 3. The release of the spring is effected by a latch. The needle of the syringe has an internal diameter of 1 mm. By means of a sleeve with sliding fit (see Fig. 3) the injector may be fixed in any desired position.

It was found to be of advantage to use absorption cells with rounded middle pieces. The needle must be immersed in the stationary fluid in such a manner that the tip is about 1 to 2

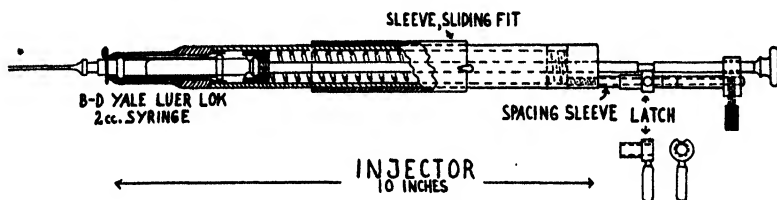


FIG. 3. Injector

mm. distant from the bottom of the cell. Whereas the beam of light may pass the trough out of center, the needle of the injector must be kept in a central and vertical position. The layer of stationary fluid above the tip of the needle must be sufficiently deep to prevent splashing owing to the back pressure of the ejected fluid. Under these conditions, the stationary fluid will cushion the shock of the injection and render it almost unnoticeable.

Testing and Calibrating of Apparatus

Determination of Mixing Time—If the stationary fluid is transparent to the monochromatic radiation selected and if a solution of a dye absorbing light of the same wave-length is injected into the cell, the time required for the galvanometer deflection to reach the level corresponding to the extinction of the final system

is a measure of the mixing time. It is assumed that the photoelectric cell, the amplifier system, and the galvanometer string introduce no appreciable inertia in the system.

Fig. 4 is the photograph of a record obtained by injecting 1.0 cc. of methylene blue solution (about 10^{-4} M) into 2.3 cc. of water as the stationary fluid in the 0.5 cm. absorption cell. The change-over from the deflection of the galvanometer, corresponding to the absorption of the water, to that of the final methylene blue solution is practically a vertical line. The time coordinates in the curve are spaced 0.04 second apart. Mixing was practically complete in this test after 0.04 second. In a number of similar experiments with different absorption cells and methylene blue solutions of varying strength, the time required for completion of mixing was found between 0.04 and 0.06 second. In these experiments the monochromator was adjusted to 630 $m\mu$. The width of the transmitted band of light was of the order of 10 $m\mu$. Methylene blue shows strong and selective absorption in this range of the spectrum.

From the tests it is concluded that reactions accompanied by changes in light absorption lasting longer than 0.05 second should be detected by the present method.

Calibration with Rotating Sector—Whereas the response of a vacuum type photoelectric cell is known to be linear under all conditions, gas-filled cells will show deviations from linearity at high light intensities. A gas-filled cell was used in the present experiments because of its greater sensitivity compared with a vacuum cell of the same design. In order to test for the linearity of response of the gas-filled cell in the range of the low intensities obtaining under the conditions of the present experiments, a rotating sector was interposed between the light source and front slit of the monochromator in the manner shown in Fig. 1. The opening in the sector disk was exactly 50 per cent of the total area. The deflection was cut down to the desired extent by means of the high resistance shunting the string of the galvanometer (see Fig. 2), whereas the voltages applied to the photocell and the amplifier tube remained unchanged. Before the sector was started, the deflections of the galvanometer corresponding to the full intensity of the light source and to the completely covered monochromator slit were recorded. Then the sector was

started, and, after it had attained full speed, a length of film was exposed. Fig. 5 shows the result in the case of a maximal deflection of 2.0 cm. The resultant produced by the sector (right-hand part of the record) shows equal distances from the extreme deflections. This proves that under these conditions the response of the photoelectric cell is linear. It is concluded that, within the range of deflections tested and in the case of solutions obeying Beer's law, changes in deflection may be considered to represent proportional changes in concentration of the compound responsible for the light absorption.

Effect of Dilution—If the stationary fluid is colored and the injected fluid is colorless, as in the following experiments, the effect of dilution resulting from the injection must be taken into consideration. Water when injected into methylene blue solutions will cause smaller or larger deflections of the galvanometer according to the degree of dilution. The dilution values are



FIG. 4. Record obtained by injecting methylene blue into water.

FIG. 5. Calibration of photoelectric cell with rotating sector.

reached after an interval comparable in length to the mixing time. If, however, a colored protein solution, *e.g.* methemoglobin, serves as the stationary fluid, the resulting change upon injection of water is rather complex. At first there is a quick deflection in the direction of less light transmission. Gradually the movement of the galvanometer string is reversed and eventually it comes to a standstill on a level corresponding to the final transmission of the diluted system. Fig. 6 is a typical record obtained by injecting 0.25 cc. of water into 2.7 cc. of methemoglobin solution. The analysis of this phenomenon has shown that the initial deflection indicative of decreased transmission is due to the formation of clouds consisting of microscopical gas bubbles. When they rise to the surface of the solution, the transmission of the system is increasing and approaches that of the final and true value. If the stationary and the injected fluids are identical in composition and concentration, the changes in light transmission upon injection are negligible.

Application of Method to Study of Catalyst-Substrate Compounds

Intermediate in Methemoglobin-Ethyl Hydrogen Peroxide Catalysis—Whereas the intermediate in the methemoglobin-hydrogen peroxide catalysis was discovered some time ago (8), the corresponding intermediate in the reaction of methemoglobin with ethyl hydrogen peroxide was only recently observed (1, 3). This reaction proceeds without gas evolution. When the peroxide is added to methemoglobin at pH 5.3, the absorption band of methemoglobin at $630\text{ }m\mu$ disappears and a compound with a band at $590\text{ }m\mu$ is formed. The rate of fading of the absorption



FIG. 6. Record obtained by injection of water into methemoglobin solution.



FIG. 7. Record obtained by adding ethyl hydrogen peroxide to methemoglobin solution at pH 5.3.



FIG. 8. Record obtained by adding ethyl hydrogen peroxide to liver catalase at pH 6.8.

band of methemoglobin at $630\text{ }m\mu$ is a measure of the transformation of free catalyst into combined catalyst.

A typical experiment was performed in the following manner: A stock solution of methemoglobin prepared by oxidizing bovine oxyhemoglobin with ferricyanide and dialyzing at low temperature was diluted to a methemoglobin content of 0.9 per cent with water and acetate buffer of pH 5.3. The final buffer strength was 0.1 M. 2.3 cc. of this solution were placed in an absorption cell of 0.5 cm. depth. Light of an average wave-length of $630\text{ }m\mu$

was allowed to penetrate the solution and fall on the photoelectric cell. The deflection of the galvanometer was small owing to the strong absorption of the solution in this range. At the experimental time of zero 0.5 cc. of 1.1 N ethyl hydrogen peroxide solution was injected and the change in light absorption was recorded on the film moving with a rate of 15 cm. per second. The experiment was carried out at room temperature (25°). The photographic record is shown in Fig. 7.

The maximum deflection in the sense of increasing transmission was reached 0.20 second after the substrate was injected. The curve is almost linear for the first 0.1 second; afterwards the slope decreases rapidly. At the maximum and during the following seconds no trace of the band in the red of free methemoglobin is detectable. The decline of the curve after the maximum is reached is due to an increase in width of the band of the intermediate around 590 $m\mu$. This absorption band eventually becomes so broad that it stretches into the region transmitted by the monochromator at a setting of the wave-length drum at 630 $m\mu$. For comparison purposes it is of advantage to determine the time required for half completion of the transformation of free into combined catalyst rather than the time required for the complete reaction. In the present experiment the half time value (t_{50}) equals 0.068 second. The maximal deflection on the original record was 3 cm.

Of a total of 52 experiments, sixteen have been selected for Table I.

The substrate was always added in considerable excess. Only a few peroxide molecules per methemoglobin molecule are actually required for the complete transformation into the intermediate (1, 3). As would be expected from a bimolecular reaction, the rate seems to depend among other factors on the volume in which the components react.

The dilution effect discussed in a previous section of this paper does not affect the position of the maximum on the time abscissa.

Intermediate in Catalase-Ethyl Hydrogen Peroxide Catalysis—The formation of a spectroscopically well defined intermediate enzyme-substrate compound was observed when a large excess of monoethyl hydrogen peroxide was added to concentrated and highly active solutions of liver catalase at neutral reaction (1).

Whereas the free enzyme shows a strong absorption band centered at $629\text{ m}\mu$, the strongest band of the enzyme-substrate compound in the visible region is at $570\text{ m}\mu$. In preliminary

TABLE I

Rate of Transformation of Free Methemoglobin into Combined Methemoglobin, with Ethyl Hydrogen Peroxide As Substrate

Average wave-length $630\text{ m}\mu$; pH 5.3; 25° .

Experiment No.	Date	Methemoglobin	Ethyl hydrogen peroxide	Maximum of light transmission reached after	Half of maximum reached after (iso)
				sec.	sec.
4	May 11	In 1.2 cm. layer.	0.7 cc. 1.1 N = 0.38	0.48	
5	" 20	15 cc. $5 \times 10^{-5}\text{ M}$	$\times 10^{-3}\text{ M}$	0.52	0.20
8		$= 7.5 \times 10^{-7}\text{ M}$		0.64	0.26
11				0.64	
12				0.64	0.26
2	June 17	In 0.5 cm. layer.	0.5 cc. 1.1 N = 0.27	0.24	0.08
3		2.3 cc. $1.6 \times 10^{-4}\text{ M}$	$\times 10^{-3}\text{ M}$	0.22	0.06
4		$= 3.68 \times 10^{-7}\text{ M}$		0.24	0.06
5				0.24	0.06
8		2.3 cc. $1.4 \times 10^{-4}\text{ M}$	0.5 cc. 1.1 N = 0.27	0.24	0.08
10		$= 3.22 \times 10^{-7}\text{ M}$	$\times 10^{-3}\text{ M}$	0.20	0.08
11				0.20	0.068
12				0.20	0.08
9	Aug. 13	In 1.0 cm. layer.	0.25 cc. 1.1 N =	0.32	0.12
11		2.7 cc. $1.5 \times 10^{-4}\text{ M}$	$0.137 \times 10^{-3}\text{ M}$	0.32	0.10
		$= 4.05 \times 10^{-7}\text{ M}$			
2	" 15	2.7 cc. $1.4 \times 10^{-4}\text{ M}$	0.25 cc. 1.8 N =	0.24	0.08
		$= 3.78 \times 10^{-7}\text{ M}$	$0.22 \times 10^{-3}\text{ M}$		

Contrary to a statement in a previous paper (2) ethyl hydrogen peroxide, like hydrogen peroxide, is bivalent as an oxidizing agent.

experiments (2) the rate of the appearance of the latter band at varying hydrogen ion concentrations and at two different temperatures has been roughly determined by direct spectroscopic observation. An attempt was made to measure by the

present method the fading of the absorption band of catalase at 629 $m\mu$ upon adding the substrate.

100 cc. of a purified liver catalase solution² of an activity of $k = 3675$ were concentrated to 10 cc. of $k = 12,180$ by the acetone procedure previously described (9). The solution was 0.033 M in phosphate buffer of pH 6.8. The concentrate showed the enzyme spectrum clearly in layers of 0.5 cm. 2.2 cc. of the enzyme solution were placed in the 1.0 cc. absorption cell. The deflection of the galvanometer corresponding to 100 per cent of free enzyme was recorded. At the experimental time of zero 0.5 cc. of 1.1 N ethyl hydrogen peroxide solution was injected. The rate of film transport in these experiments was 4.2 cm. per second. One of the records obtained is shown in Fig. 8.

Control experiments have shown, however, that the initial phase of the enzyme reaction is obscured by foaming, probably owing to the rapid decomposition of traces of hydrogen peroxide present in the ethyl peroxide preparation, resulting in the evolution of oxygen. An attempt is being made to overcome this difficulty by the use of an apparatus containing two photoelectric cells in a compensating circuit. For the present, no conclusions are drawn concerning the kinetics of the enzyme-substrate reaction.

DISCUSSION

The rate of formation of the methemoglobin-ethyl hydrogen peroxide compound is smaller than the rate of combination of reduced hemoglobin or hemocyanin with oxygen. According to Millikan's measurements obtained by the flow method (10), the time required for half completion of oxyhemoglobin formation (sheep) is 0.01 second and for the corresponding reaction of *Limulus* hemocyanin 0.008 to 0.012 second. The rate of formation of the methemoglobin-peroxide intermediate appears to be of the same order as that of the dissociation of oxyhemoglobin or oxyhemocyanin into oxygen and the reduced pigment ($t_{50} = 0.025$ to 0.05 and 0.025 to 0.1 second respectively (10)). However, more data are required before a more detailed comparison can be made.

² The authors are indebted to The Hill Packing Company, Topeka, for supplying the frozen horse liver used for these preparations.

SUMMARY

A method has been developed for the kinetic study of chemical reactions which are accompanied by changes in light absorption. One of the reactants is kept stationary, whereas the other is rapidly added from an injector mechanism. Mixing is complete within 0.04 to 0.06 second. The system under study is placed between a monochromator and a photoelectric cell. The photoelectric current is amplified and the changes in light absorption occurring after the reactants are mixed are recorded by means of a string galvanometer and a film camera. By varying the speed of film transport reactions of different rate may be studied. The method is not meant to replace but to supplement the flow method of Hartridge and Roughton, which permits the study of considerably faster reactions.

The method is applied to the study of the formation of catalyst-substrate compounds. The reaction between methemoglobin and ethyl hydrogen peroxide leading to the formation of an unstable intermediate is half complete within 0.06 to 0.26 second, according to the experimental conditions selected. The reaction appears to be somewhat slower than the rate of formation of oxyhemoglobin or oxyhemocyanin; it appears to be of an order similar to the dissociation of these complexes.

The method in its present form is adequate for the study of simple systems. For a satisfactory analysis of the catalase-peroxide reaction it will require further refinement.

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POTENTIOMETRIC STUDY OF THE FLAVINS

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The yellow dyestuff which, in the form of its phosphate ester and combined with a particular protein, forms Warburg's yellow respiration ferment (1) is a derivative of isoalloxazine (Formula I, with $R = H$). This was first suggested by Stern and Holiday (2) and definitely proved by Kuhn *et al.* (3, 4) and Karrer *et al.* (5). This dyestuff has been identified by György, Kuhn, and Wagner-Jauregg (3) with vitamin B_2 , or better, with one of those substances which were formerly, as a group, designated as vitamin B_2 . The vitamin is 6,7-dimethyl-9-*d*-riboisoalloxazine. Isoalloxazine itself is tautomeric with alloxazine in which the H in position (9) is shifted to position (1), and seems to exist to the greater extent in the latter form. In the substituted compounds no ambiguity exists, so we shall omit the prefix iso. The present studies are concerned with alloxazine, 9-methyl alloxazine, 9-*d*-glucoalloxazine, and with four 6,7-dimethyl derivatives, which will be designated as flavins, namely 9-methyl flavin (lumiflavin), 9-*d*-glucoflavin, 9-*l*-araboflavin, and 9-*d*-riboflavin (vitamin B_2). All these dyes were prepared synthetically according to Kuhn's method.

The similarity of the structure of these compounds to those of the phenazine group, especially to pyocyanine, is obvious. Accordingly, they share with these dyes the ability of being reversibly reduced and the ability to form, on partial reduction in acid solution, an intermediate form (6), referred to as the *S* form, half-way between the reduced, or *R* form, and the totally oxidized, or *T* form. Kuhn and Wagner-Jauregg (7) were the first to show experimentally this analogy of lactoflavin and pyocyanine. Any alloxazine or flavin dye, dissolved in strong HCl, passes on reduc-

tion from the original intense yellow through red to a pale yellow. In analogy with the interpretation previously applied to the phenazine compounds by Michaelis and Hill (8), Kuhn and Wagner-Jauregg considered the red form as a semiquinoid radical. This assumption is justifiable as in all comparable cases the intermediate form is of this character and never a bimolecular meriquinone.

The formation of the red *S* form is very striking at $\text{pH} < 0$, but decreases rapidly at lesser acidity. At $\text{pH} > 1$ no intermediate color has been described as yet. Even at $\text{pH} 1$, only a trace of orange-pink can be seen. This, however, does not exclude the possibility of a small amount of the *S* form being present even where it cannot be directly recognized by a color change. The case of pyocyanine (6) suggests that this may be so. Here it could be shown that a small amount of the *S* form can exist in equilibrium with *T* and *R* forms throughout a wide pH range in which a color change was not distinctly manifested. The evidence was obtained by an analysis of the potentiometric titration curves. It will be an essential part of this paper to decide whether and to what extent this is also the case for the dyes here investigated.

There are good reasons why the formation of a small amount of the *S* form may be concealed as far as color observation goes. The color of the *S* form may not be different enough from that of the others to be noticeable when present in only a small percentage. Certainly, the red color of this semiquinone is very striking when one is working with 2 *N* HCl and with a rather concentrated solution of the dye. Under proper conditions it crystallizes in large red plates (not yet described by any previous author) differing greatly from the yellow crystals of the *T* form and the thin long, pale yellow prisms or needles of the *R* form. But even at this low pH , in high dilution the color of the *S* form is much less striking and, being mixed with the yellow, results in only a faint orange. Furthermore, it is quite possible that at higher pH the *S* form is in another state of ionization and has a different color. We shall show that this is actually the case. We shall see that different states of ionization of the *S* form exist at different values of pH . The one obtainable at $\text{pH} < 1$ is red, the one at any $\text{pH} > 2$ is green. The contrast of this green color with yellow, although very striking under favorable conditions, as will

be shown, is very small under ordinary conditions, and so has escaped the attention of all previous authors. This color change, observable during reduction, leaves no doubt that an intermediate form exists to a small extent even at higher pH.

The opinions of previous authors as to the existence of a semi-quinone at higher pH, where no red color can be observed, are divergent. Bierich and Lang (9) report index potentials of 14.6 to 14.7 millivolts. They take them, within the limits of error, to be 14.3 and indicative of a one-step bivalent system. Stepwise reduction was not known to these authors. Stern (10), in an elaborate paper on this subject, reports for hepatoflavin and lumiflavin prepared from yeast index potentials greater than 14.3 millivolts throughout the whole experimental pH range. In full recognition of this author's results we believe that at the present time, when the material can be prepared in larger amounts, the quantitative side of this problem should be elaborated with greater precision. We shall see that the picture then obtained becomes compatible with the use of all the ionization constants imaginable according to the structural formulæ of the dye. This is not obvious from Stern's curves, although qualitatively he was right as to the general form of the curves. Kuhn and Moruzzi (11) consider the index potentials at all pH values not showing the red intermediary form to be, within the limits of error, those of a one-step bivalent system. According to Barron and Hastings (12) the steps in neutral or weakly acid solutions overlap so much that the curves are "similar to curves with a 2 electron transfer." Stare (13) arrives, qualitatively, at a result similar to Stern's. He reports, however, in an intermediate pH range an index potential of about 28 millivolts, which is much higher than any obtained by Stern or by the writers. None of these authors was aware of the existence of the green intermediate form.

Mean Normal Potentials, E_m

The normal potential, E_m , of a mixture consisting of equal parts of *T* and *R*, corresponds to the normal potential, E_o , of a regular organic dye. Fig. 1 summarizes these normal potentials as obtained experimentally. Table I gives some data for the physiological pH range. The data, for not too acid solutions, agree satisfactorily with those of previous authors. It can be

seen that there is no noticeable difference among the three flavins with sugar side chains, but that there is a distinct difference between glucoflavin and glucoalloxazine. Furthermore, there is a difference between the methyl compounds and the sugar compounds both in the alloxazine and the flavin series. The most negative potential is that of alloxazine itself. The study of this compound meets particular difficulties, in part due to its very slight solubility, especially in its reduced form. For this reason we restrict ourselves for the time being to plotting only one point in the diagram, Fig. 1.

There is a dissociation constant of the oxidized forms of all the compounds, $pK_1 = -0.2$ approximately; and another $pK_2 = 9.8$. The first is obviously due to the very weak basic property of the N atom in either position (9) or (10), of Formula I; the

TABLE I
Normal Potentials at 30°

pH	5	6	7	8
Glucoalloxazine.....	-0.072	-0.125	-0.163	-0.193
Methyl alloxazine.....	-0.092	-0.145	-0.183	-0.213
Gluco-, arabo-, and riboflavin....	-0.117	-0.170	-0.208	-0.238
Methyl flavin (lumiflavin).....	-0.132	-0.185	-0.223	-0.258
Alloxazine.....	-0.170			

second concerns the acid property of the one CO—NH group of the alloxan ring. For the *R* form, there is one $pK_1 = 6.1$, due to the OH group in position (4) (Formula VI), the acidic property of which is strengthened by the CO group in the same ring. Due to this acid group, the acidic property of the CO—NH group itself is so much weakened that in the pH range investigated no further pK_1 is found that might correspond to $pK_1 = 9.8$. There is no evidence for a pK_2 analogous to the $pK_1 = -0.2$. Obviously, the NH in position (10) of the *R* form is still less basic than the N in the same place of the *T* form. This recalls the extremely weak basic property of diphenylamine.

The color of the *T* form is very pale yellow at negative pH values; it is intensely yellow with a greenish fluorescence between pH 0 and 9; it is intensely yellow without fluorescence at pH > 10.

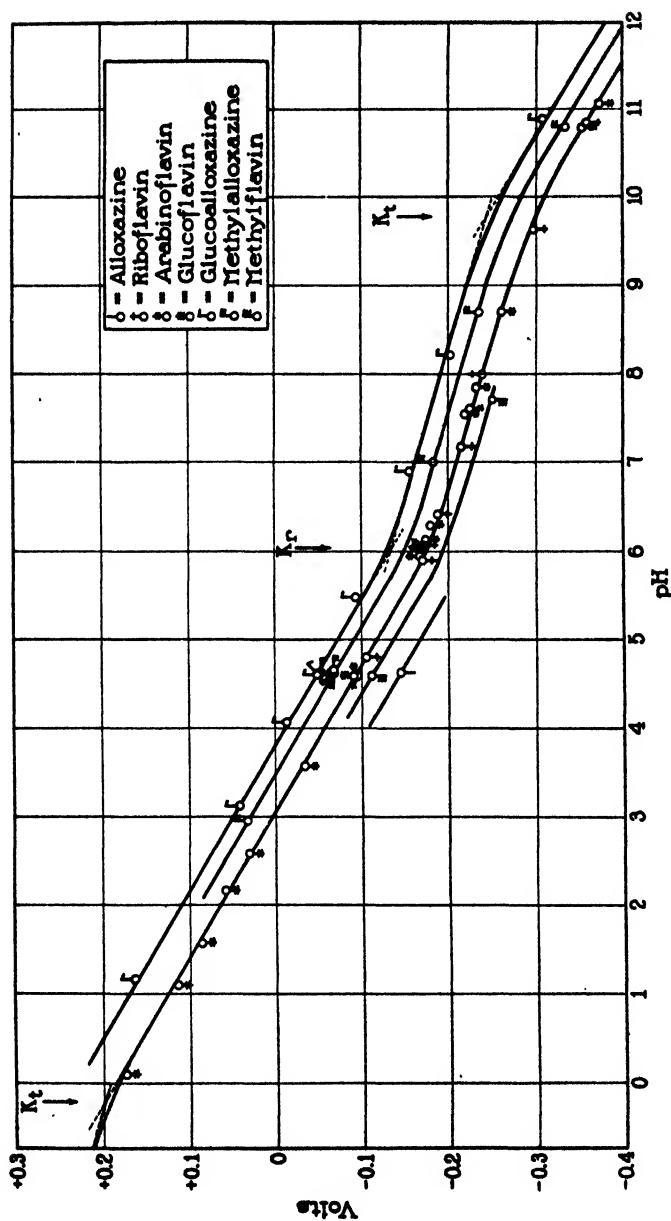


Fig. 1. Normal potential, E_m , referred to the normal hydrogen electrode, plotted against pH. The buffers used were HCl, lactate, acetate, phosphate (in its two ranges), veronal, dimethylglycine.

The *R* form is very pale yellow, with a slight greenish hue without fluorescence at pH up to 6, and somewhat more intensely yellow at higher pH. The *R* form is much less soluble than the *T* form, and even in its ionized form, at pH > 7, crystallizes easily in pale yellow, long needles. It is very much more soluble at higher temperatures.

E₁ and E₂ Normal Potentials

The normal potentials of the *R*, *S* system, E_1 , and those of the *S*, *T* system, E_2 , have been determined by the method previously elaborated (14). The calculations are based on the experimental determination of the index potential E_i , i.e. the difference between the potential at 25 per cent (or 75 per cent) and 50 per cent reduction.

Considering all of the numerous experiments, we have never found the index potential at pH 3 to 10 and higher to be less than 14.8 millivolts or greater than 17 millivolts. The scattering of the individual results around the average value is evenly distributed among the various representatives of this group of dyestuffs and throughout this pH range. Therefore, we believe that the index potential is the same, within the limits of error, throughout the mentioned pH range and for all the dyes investigated. To arrive at the most probable value, however, we did not think it proper to average all the experiments and have selected the last set obtained, since experience with the methods had resulted in a considerable improvement of them. The results are given in Table II.

The normal potentials, E_m , E_1 , E_2 , for the flavins have been plotted in Fig. 2. That part of the curve at pH < 1 was not arrived at by the potentiometric method, for reasons to be discussed later, but has been extrapolated on the basis of a spectrophotometric determination of $pk_i = -0.2$. There is a $pk_s = 1.3$, another $pk_s = 8.0$. The first of these is analogous to $pk_i = -0.2$; the second is analogous to $pk_r = 6.1$, or to $pk_i = 9.8$.

The order of the pk values in the neighborhood of neutrality is most understandable if they are considered as due to ionization in the alloxazine ring. The acidic property decreases in the order *R*, *S*, *T*. It is easy to see why *R* stands before *T*; and it is acceptable that *S* stands midway between them. It is not so easy to

account for the order in strongly acid conditions. It is, however, important to state that in all comparable cases studied so far, pyocyanine, α -oxyphenazine, and chlororaphine, the same situa-

TABLE II
Index Potentials at Various pH Values

	pH	E_i millivolts
Methyl flavin	4.62	17.0
Glucosflavin	4.62	15.8
	10.81	15.6
Riboflavin	4.62	17.0
	4.62	16.3
	4.83	15.7
	5.38	16.4
	6.38	16.5
	7.10	16.0
	8.07	16.0
Average.....		16.2

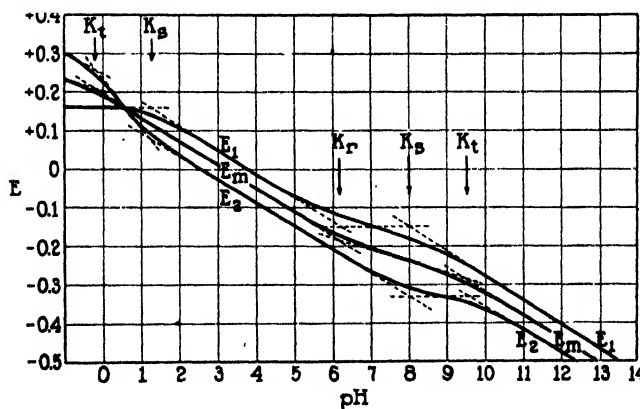


FIG. 2. The three normal potentials E_1 , E_m , E_2 of riboflavin plotted against pH.

tion obtains: pK_4 is a little larger than pK_3 , but there is no pK_4 in this neighborhood to which the pK_4 could be related. This fact is explainable; the argument should, however, be postponed to a later paper on this problem.

One interesting problem, perhaps the most important of all, is this: What fraction of the total dye can exist *in maximo*, in the *S* form, in equilibrium with the *R* and *T* form? This maximum amount obviously always exists at 50 per cent reduction. It has been shown in a previous paper (15) that this maximum value equals $\sqrt{K}/(\sqrt{K} + 2)$, where *K* is the effective formation constant of the semiquinone, which can be calculated from the index potential. The result is that throughout the physiological pH range, even between pH 2 and 12, about 10 per cent of the total dye can exist as a semiquinone at the proper state of reduction (*i.e.* at 50 per cent reduction). The limits of error in this figure, due to errors in the underlying data, are relatively small and will be properly discussed later on.

Since the property of forming a semiquinone in the physiological pH range is very rare among the dyestuffs, it is quite possible that this particular semiquinone is the "active" form of the dye functioning as ferment or vitamin. This is for the time being only a speculation which needs further test by experimentation.

EXPERIMENTAL

Preparation of Dyestuffs—The isoalloxazines and the flavins (*i.e.* 6,7-dimethyl isoalloxazines) were all prepared by the boric acid condensation method of Kuhn and Weygand (16). The products were recrystallized by dissolving in dilute NaOH, filtering, and reprecipitating with acetic acid, care being taken to conduct these operations in the dark. In all cases the recrystallized products on microscopic examination appeared as bundles of bright yellow needles. The yields usually were about 60 per cent. In Table III are given the analytical data and the melting points, or rather decomposition points, of all the compounds.

The intermediate compounds were all prepared by methods which can be traced through the papers of Kuhn and his associates. *l*-Arabinose was prepared from cherry gum. *d*-Arabinose from calcium gluconate was converted to *d*-ribose by the method of Austin and Humoller (17). The *d*-ribose had a melting point of 86–87°. The only step offering any difficulties was the reduction of the sugar oximes to the corresponding amines. The yields with sodium amalgam reductions are so small that catalytic reductions with palladium and hydrogen were tried. In the case of arabinos-

oxime the arabinamine was obtained in crystalline condition but in a yield not much better than that resulting from the amalgam reduction, while in the case of ribosoxime only traces of an oily product were secured, so that finally the sodium amalgam reduction was used.

The *l*-arabinamine had a melting point of 128–129°, and the *d*-glucamine 126°. The *d*-ribamine was not obtained crystalline. 4,5-Dinitroxyline, prepared by the method of Crossley and Renouf (18) (m.p. 114°) was condensed with these sugar-like amines by heating in 80 per cent alcohol in sealed tubes for 5 hours. The condensation products were all orange, crystalline

TABLE III
Analyses of Various Dyestuffs

	Formula	N calculated	N found	M.p. (corrected) °C.
Alloxazine.....	C ₁₀ H ₈ O ₂ N ₄	26.16	26.29	
9-Methyl isoalloxazine.....	C ₁₁ H ₈ O ₂ N ₄	24.56	24.51	360
9- <i>d</i> -Glucisoalloxazine.....	C ₁₆ H ₁₈ O ₇ N ₄	14.81	14.62	299
9-Methyl flavin.....	C ₁₅ H ₁₂ O ₂ N ₄	21.88	22.12	340
9- <i>d</i> -Glucoflavin.....	C ₁₈ H ₂₂ O ₇ N ₄	13.78	13.45	250
9- <i>l</i> -Araboflavin.....	C ₁₇ H ₂₀ O ₆ N ₄	14.88	15.96*	303
9- <i>d</i> -Riboflavin.....	C ₁₇ H ₂₀ O ₆ N ₄	14.88	14.56	284

* The N value is too high in this preparation. For this reason, for those measurements claiming the highest precision obtainable, as presented in Table II, araboflavin is not included.

compounds. Lumilactoflavin, as well as 9-methyl isoalloxazine, was prepared synthetically by the method of Kuhn and Reine-mund (4) rather than by photolysis of a 9-sugar flavin, since Karrer *et al.* (19) have shown that photolysis of lactoflavin gives lumichrome as well as lumiflavin.

Potentiometric Method

These dyestuffs are not quite as easy to handle with respect to potentiometric, oxidative, or reductive titration as many others. The more important representatives, *i.e.* the sugar derivatives, are liable to be decomposed if not properly treated. They are, according to Warburg and Christian (20), sensitive to light, espe-

cially in alkaline solution. One breakdown product of a flavin is lumiflavin (methyl flavin), and another is unsubstituted flavin (lumichrome) according to Karrer *et al.* (19). These two dyes have normal potentials somewhat different from that of the original dye, but the difference, however, is so small that the dyes when present in small percentage cannot be distinctly separated in a titration curve. The curve will become asymmetric about its middle point, but not necessarily to such a degree as to be distinct. Such an occurrence would result in an error in the determination of the index potential. The dyes used should be not only quite pure from the outset, but also well protected from light during all operations. The solutions were made up in dim light and during the titration the vessel was wrapped in black paper.

Furthermore, the solubility of the dyes is rather slight. This is especially true for the reduced form, so that during a reductive titration the reduced form is apt to crystallize out in fine long needles unless the concentration is very small. Fortunately, platinum electrodes respond very promptly and reproducibly even to extremely dilute solutions. The concentration finally chosen for the experiments was 2 mg. of flavin dissolved in 50 cc. of buffer, *i.e.* about 3×10^{-4} M. For methyl flavin (in which 1.7 mg. is the equivalent amount) even this concentration is too high to avoid partial precipitation at the end of a reductive titration, at 30°.

The necessity of working in so low a concentration causes a certain difficulty in determining the true 0 point of titration. In a reductive titration the oxygen should be entirely bubbled out before the first portion of the reducing agent is added. As the elimination of oxygen by displacement with nitrogen is not only slow but also asymptotic, it may cause difficulties with very dilute solutions, recognizable indeed in the titration curve, but only after the experiment has been finished. We adopted the following method. No attempt was made to bubble out the oxygen entirely, but the remainder of oxygen was titrated out with the reductant. This was added in very small steps and with ample time between these steps. In this way the 0 point could be graphically determined with great precision (Fig. 3). This method was so satisfactory that it should be recommended generally. In an oxidative titration the hydrogen can be titrated out by the oxidative reagent

in the same way. The use of such a low concentration of the dye safeguards any noticeable change of pH during the titration. This is important especially in this kind of work, where a change of pH obviously would falsify the index potential.

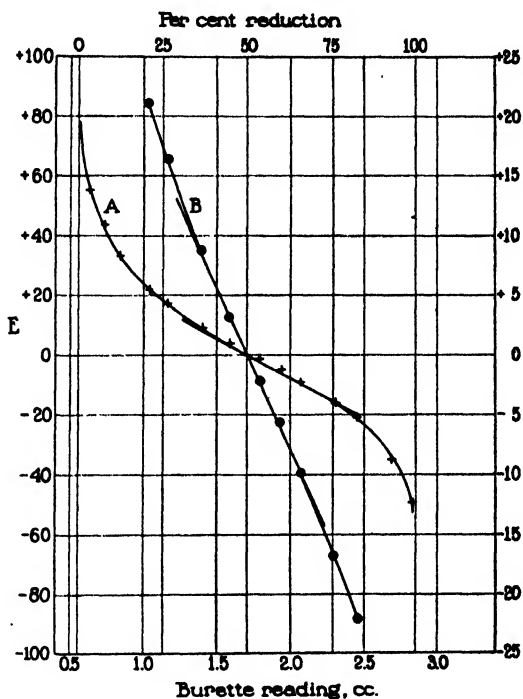


FIG. 3. 2 mg. of riboflavin, dissolved in 50 cc. of acetate buffer titrated with a solution of sodium hydrosulfite, 12 mg. in 20 cc. of H_2O . pH of buffer before titration (glass electrode) = 4.62; pH of the solution after finishing the titration = 4.62. The abscissa represents, lower scale, reading of burette in cc.; upper scale, per cent of reduction. The ordinate, left-hand scale, to be used for Curve A represents potential, referred to the normal potential, which is -0.0939 volt; the right-hand scale, to be used for Curve B, is 5 times enlarged. Curve A shows the graphic determination of the 0 per cent and 100 per cent point of reduction. Curve B shows, on an enlarged scale, the graphic determination of E_t .

The most satisfactory method was the reductive titration with hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$), not only in alkaline but also in acid solutions, in contrast to the experience of some authors with other

dyestuffs. 12 mg. of sodium hydrosulfite were dissolved in 20 cc. of water containing a small drop of 0.1 N NaOH. The addition of the alkali prevents any decomposition of the hydrosulfite. Without this precaution it happens once in a while that the hydrosulfite solution even if perfectly protected from oxygen, after being transferred to the burette, becomes slightly turbid after a short time owing to formation of sulfur. This never occurs after the alkali is added. The amount of alkali is not great enough to bring about any measurable change of pH in the well buffered dye solution as has been frequently checked by the glass electrode. (The hydrogen electrode cannot be used in a solution containing hydrosulfite.) The details of preparing this solution are as follows. To avoid mixing, the solid hydrosulfite was put into a container immersed in the alkaline water. After bubbling with nitrogen the container was tipped over, and the hydrosulfite dissolved and forced into the burette by nitrogen pressure. Then the dye solution was poured into the titration vessel, bubbled with nitrogen for only 5 to 10 minutes, and the remaining oxygen titrated out very slowly as described above. Each portion of hydrosulfite was added while the titration vessel, held in a clamp, was agitated by revolving it by means of a motor, about a perpendicular eccentric axis.

This method can be used for all $\text{pH} \geq 2$. At smaller pH, no good end-point can be recognized, obviously due to an overlapping of the potential range of the dye and the reductant. Besides, especially at $\text{pH} < 1$, the establishment of the potentials is no longer prompt and not quite reproducible at various electrodes.

Not all dyestuffs, to be sure, can be safely titrated with hydrosulfite quite as satisfactorily. The oxidation product of hydrosulfite is sulfite. If the dye be of such a positive potential range that it can be reduced by sulfite, or can react with sulfite in any way, hydrosulfite gives no perfectly stable potentials. This, however, is not the case with the flavin dyes.

Other reducing agents have been tried, especially chromous acetate. As far as tested, in moderately acid solutions, the results agree with those with hydrosulfite, but no advantage could be seen. At $\text{pH} < 1$, the end-points of titration were no better than those with hydrosulfite. So, for pH 0 to 2, the oxidative titration with benzoquinone was chosen, after reducing the dye with the

smallest possible amount of colloidal palladium in a stream of hydrogen. In that small pH range around 2, where both the oxidative and the reductive method could be used, identical results were obtained. In general, titrations at $\text{pH} < 1$ are not of much avail as the determination, even the definition, of pH is somewhat uncertain. They just show qualitatively that the separation of the two steps of oxidation is more distinct than at higher pH.

Direct Evidence of Existence of Intermediate Form. Its Red and Its Green Modification

As an example of how to demonstrate the green color of the *S* form at pH 2 to 12, the following experiment is described. A solution of any substituted alloxazine or flavin¹ in, say, an acetate or phosphate buffer, nearly saturated with the dye at about 70–80° and permanently kept about this temperature, is mixed with a proper amount of solid sodium hydrosulfite. The color will change from bright yellow through a dark olive-brown, to a more or less distinctly green shade according to conditions, finally to pale yellow. On being shaken with air, the temperature always being kept high to avoid precipitation, the color change will be reversed, and will pass through the same stages. It will not be decided for the time being whether the great ease with which the *S* form can be detected in this way is simply due to the higher concentration used or also to an increase of the formation constant of the semiquinone with increase of temperature. At any rate, the success of this qualitative experiment increases our confidence in the results obtained from potentiometric data of such a delicate nature that anyone mistrusting the technique of the authors would be inclined to take them as artifacts.

On performing such an experiment with a buffer of $\text{pH} > 10$ the solubility of the *T* form is so high that a solution concentrated enough for the purpose can be obtained without heating. The *R* form, however, is even here so little soluble that it may precipitate unless heated.

At room temperature the concentration attainable is so low that the color change is not so obvious. It can, however, be observed

¹ The unsubstituted parent substance is so little soluble even on heating that the observation is much more difficult, but can be made.

by looking through the whole length of the test-tube. The fact that the formation of the intermediate form can be seen at all in higher dilution is a further evidence that it is a semiquinone radical and not a bimolecular meriquinone as has recently been pointed out for a similar case. We refer to the "dilution test" (15).

Methods Used in Construction of Fig. 2

The E_m curve of riboflavin is redrawn from Fig. 1.

Between pH 2 and 5 the index potential is taken as 16.2 millivolts. From this, according to a graphic interpolation from Michaelis' Table I (6) as basis, or by applying Elema's formula (21) (reproduced by Michaelis (6) as Equation 18) we obtain for the effective formation constant of the semiquinone, K , the value 0.11. From this, $E_2 - E_1$ or its half, $E_m - E_1 = E_2 - E_m$, can be calculated, according to Equation 8 of Michaelis (6), to be 0.072 volt. In this way, the three potential curves for pH from 2 to 5 are obtained.

At pH 6.1, there is a bend in the E_m curves corresponding to a k_r . So, the E_1 and E_2 curves should diverge from this pH, and E_i should rapidly converge to its minimum value 14.3 millivolts as pH increases. The fact that this expectation is not confirmed by the experimental data indicates the existence of an acidic dissociation constant of the S form, k_s , which reestablishes the parallelism of the three curves. The approximate magnitude of this k_s can be inferred as follows: Even at pH > 10 we find the values of the index potential within the limits of error the same as before. This determines the distance $E_1 - E_2$ such as drawn for the pH range 10 to 12. Now, there is a $pk_i = 9.5$ as shown by the bend at pH 9.5. This results in a bend in the E_2 curve, but in no bend in the E_1 curve. The intersection of the lines, preliminarily drawn as straight lines, gives $pk_s = 7.8$. For the establishment of the real course of the curve, the angles of intersecting tangents need only be properly rounded out to account for the overlapping in the transition zones. This rounding out may be assisted by the following rules which can be easily derived from the theory.

At the pH of the point of intersection, the difference of the ordinate at this point and the ordinate of the corrected curve should be 0.018 volt for a univalent system, or 0.009 volt for a bivalent one.

If two bends follow at a small distance from one another, as is the case for the E_1 curve around pH 7, the overlapping will cause even a more gradual change, a diminution of all curvatures.

Let us now discuss the consequences of any error in the index potential. Table IV will show the consequences.

*Approximate Spectrophotometric Determination
of $pK_i = -0.2$*

This determination is based on the fact that a flavin, intensely yellow in neutral solution (with greenish fluorescence) undergoes a reversible color change in a strongly acid solution to a much lighter yellow (without fluorescence). The disappearance of

TABLE IV
Consequence of Error in Index Potential

E_i	Formation constant K	$E_2 - E_1$	Maximum ratio of semiquinone to total dye, M
0.0156	0.029	-0.092	0.078
0.0159	0.044	-0.081	0.095
0.0162	0.0625	-0.072	0.111
0.0165	0.078	-0.064	0.122
0.0168	0.108	-0.058	0.148

The following equations were used in the calculation. (1) $K = (a - (3/a))^2$, where $a = 10^{0.06601}$ according to Elema (21); (2) $E_2 - E_1 = 0.0601 \log K$ according to Michaelis (14); (3) $M = \sqrt{K}/(\sqrt{K} + 2)$ according to Michaelis (15). So it can be seen that the consequences of an error even of ± 0.6 millivolt in E_i are not conspicuous. M remains of the order 0.1.

fluorescence has already been used for a determination of k_i by Kuhn and Moruzzi (22). Quantitatively, their result, $pK = 2.0$, does not agree with the one presented here ($pK = -0.2$), although we cannot claim a great accuracy for our value. The disagreement may be due in part to an unknown intrinsic error in the photometry of fluorescence. We measured the light absorption at wave-length 4950 Å. with a König-Martens spectrophotometer, comparing the absorption of water and that of a solution of the dye in a final concentration of 0.02 per cent made up in water or HCl solution of varied concentrations. The difficulty of definition of pH in strong HCl solutions, and other, optical, limitations of the method make it seem not worth while describing

the experiments in detail. Assuming that in pure water the dye is entirely un-ionized, and in nearly concentrated HCl 100 per cent ionized, we obtain the result given in Table V.

These figures show that somewhere between 1 and 2 M HCl, say at pH around -0.2 , the ionization is 50 per cent, and so we use the value $pK_i = -0.2$, without claiming much accuracy for it.

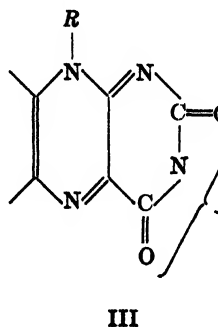
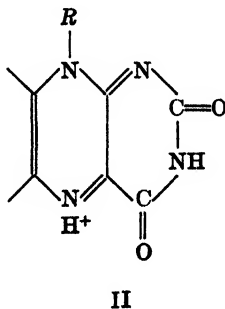
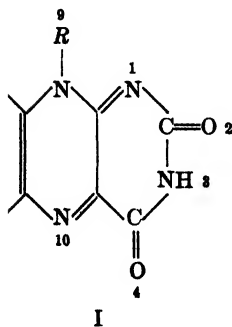
9-l-Araboflavin gave essentially the same result.

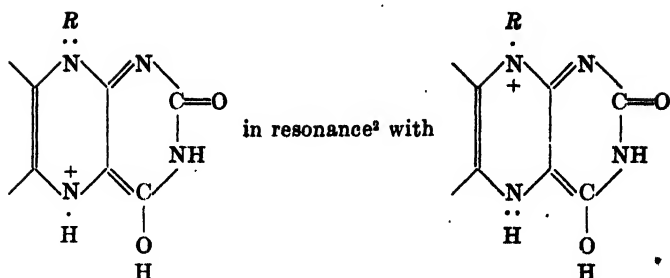
TABLE V
Degree of Ionization in Strongly Acid Solution, Spectrophotometrically Determined, for Riboflavin

HCl concentration	Degree of ionization
<i>M</i>	
0	0.00
0.11	≈ 0.04 Approximate
0.21	0.04
0.50	0.12
1.05	0.31
2.10	0.84
10.7	1.00

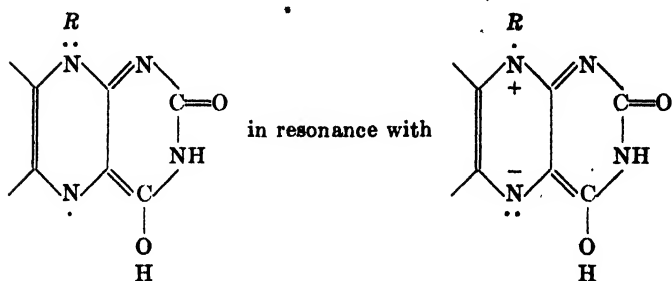
Structure of Dye in Its Various Forms of Oxidation-Reduction and Ionization

We assume Formula I as the structure of a flavin in a neutral solution in its *T* form. Then in a solution of negative pH we will have Formula II, and at pH > 10 , we have Formula III. To establish the structure of the other forms we may utilize the

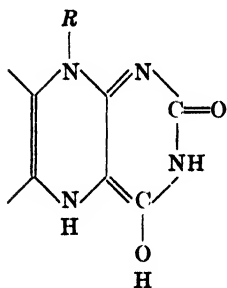




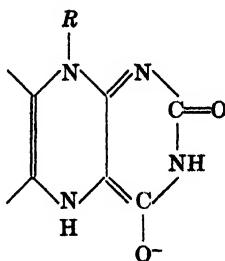
IV



V



VI



VII

diagram, Fig. 2, and apply the following rules. At such a pH range where the slope = 0, the oxidized and the reduced forms of the system concerned differ by 1 electron for a univalent system,

² This term, taken from the terminology of quantum mechanics, signifies a periodic oscillation between two electronic structures. The energy of this oscillation may be thought of as supporting the stability of such a molecule, which may be and is really in our case a radical and may be incapable of existence otherwise.

or by 2 electrons for a bivalent system. When the slope = 0.06 volt per pH unit, the difference is 1 hydrogen atom for a univalent system, or 2 hydrogen atoms for a bivalent system. A 0.03 slope can occur only in a bivalent system and then the difference is 1 electron and 1 hydrogen atom (or 2 electrons and 1 proton). The E_m curve refers to a bivalent system, the E_1 or E_2 curve to univalent ones. We thus obtain for the S form, at pH < 0 (the red form), Formula IV, in which the points are electrons. The number of all electrons is odd.

1 electron oscillates between the 2 N atoms, not by a direct jump through space, but by the intermediation of the conjugated carbon chain. At pH 2 to 7, the S form has 1 proton less, and can be pictured as Formula V. This is the green form of the semiquinone. At pH > 8 another step of dissociation occurs, due to the ionization of the OH group in the alloxan ring. The resonance is of the same character as in Formula V, and so is the color; both are green. No further step of dissociation of the S form is imaginable, as the remaining NH—CO group is necessarily of extremely weak acid character in a ring already negatively charged. The R form does not exist, within the limits of the pH range used, in a state of ionization analogous to Formula II. It exists, at pH from negative values almost up to 6, in the form of Formula VI, and at higher pH forms in the ion, Formula VII. There is no great change of color of the material of Formula VI to that of Formula VII; Formula VII represents somewhat more intensely yellow material than does Formula VI. No further ionization in the alloxan ring is observed even at the highest pH of its experimental range.

These considerations show that the ionizations inferred from the potentiometric experiments can be readily fitted into the structural picture. No more and no fewer states of ionization can be inferred than can be reasonably expected from structural considerations.

Effect of Flavin and Alloxazine Dyes on O_2 Consumption and Fermentation of Yeast Extract

It is clear from the experiments of Warburg and Christian (23) that in their system their yellow dyestuff (flavin) does not function as an oxygen (or hydrogen) transporter until it is combined.

with phosphoric acid and a particular protein. However, the flavins are readily autoxidizable, reversible dyestuffs, so it is to be expected that in systems which are sufficiently strongly reducing they will act as catalysts of oxygen consumption. It has been shown by Wagner-Jauregg (24, 25) and his coworkers that some, but not all, dehydrogenase systems that readily reduce methylene blue will also reduce flavin. They also report (24) experiments by N. Brookens showing that purified ovoflavin solutions do not increase the O_2 consumption of red blood cells. It is to be expected (26) that dyes with such negative potentials will not increase the respiration of red cells. Yeast extracts are known (27) to be more strongly reducing, so we have tested the effect of the

TABLE VI
Effect of Dyes on O_2 Consumption

Dye added	O_2 consumed after 145 min. at 30°		
	Concentration of dye	Substrate = 0.4 M glucose	Substrate = 0.02 M added CH_3CH_2OH
	M	c.mm.	c.mm.
None.....		63	34
Lumilactoflavin (methyl flavin)....	1×10^{-4}	95	42
Methyl alloxazine.....	1×10^{-4}	306	99
Glucoflavin.....	1×10^{-4}	93	45
Glucoalloxazine.....	1×10^{-4}	179	89
Araboflavin.....	1×10^{-4}	93	51
Riboflavin.....	1×10^{-4}	103	43

above dyes on the O_2 consumption of Lebedev extract, using both glucose and alcohol as substrates. Some results are shown in Table VI. It is clear that the alloxazines have a much greater effect than the flavins. Whether or not this difference is entirely due to the slight difference in their normal potentials is not clear, but it would seem unlikely since the methyl alloxazine, though it has the strongest effect, is more negative than the glucoalloxazine.

The dyes listed in Table VI were also tested for their effect on aerobic fermentation with the same extracts. A slight acceleration of the beginning stages of fermentation, especially in the case of the two alloxazines, was observed followed by a slight inhibition.

SUMMARY

The normal potentials of a number of derivatives of isoalloxazine and flavin, including what is called lactoflavin or vitamin B₂, are reported and plotted against pH. All these dyes are capable of forming, on partial reduction, a semiquinone which is red in very acid solution and green at pH > 1. The formation constant of the semiquinone and the maximum amount of semiquinone capable of existence in equilibrium with the other forms of the dye are calculated. This maximum amount, through a wide pH range including the physiological pH range, is about 10 per cent. The course of the E_1 , E_2 , and E_m curves is determined by the various acidic dissociation constants of the dye in its three states of oxidation-reduction. The relationship of these constants is such as to bring about a parallelism of these three curves over a wide pH range, involving the consequence that the maximum percentage of semiquinone capable of existence remains practically constant over a pH range from 1 to 12.

Among these dyestuffs the naturally occurring flavins increase the oxygen consumption of yeast extract only very slightly, very much less than methylene blue. The physiologically non-occurring alloxazine dyes are more effective in this respect and comparable to methylene blue.

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THE DETERMINATION OF ASCORBIC ACID AS FURFURAL AND A COMPARISON OF RESULTS OBTAINED BY THIS METHOD AND BY INDOPHENOL TITRATION

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In seeking for a chemical procedure, other than oxidation-reduction measurement, that could be made the basis of a quantitative method for the determination of ascorbic acid, it was observed that this substance forms furfural when boiled with HCl. As furfural can be determined readily by appropriate treatment with aniline acetate, this reaction was made the basis of a quantitative method for the estimation of ascorbic acid.

Reversibly oxidized ascorbic acid does not yield furfural when boiled with HCl. Advantage is taken of this fact and of the easy oxidizability of ascorbic acid to develop a specific method. The method consists essentially of the determination of the furfural obtained by boiling an oxidized extract of a tissue with HCl under reducing and non-reducing conditions. The value obtained with reducing conditions minus that produced under non-reducing conditions is the amount of furfural from ascorbic acid.

An important reagent in this procedure is norit, which is used for two reasons. Norit is a highly efficient clarifying agent, removing interfering pigment from acid extracts of any plant or animal tissue. Norit also oxidizes ascorbic acid to dehydro-ascorbic acid. The latter is adsorbed to some extent on norit in neutral or slightly acid ranges, but is quantitatively eluted in a solution containing 10 per cent, or more, of acetic acid.

The color formed when furfural is treated with aniline is destroyed by an excess of aniline and by oxidation, the oxidative effect of the atmosphere being marked. Aniline excess is controlled by the addition of enough glacial acetic acid to bring the

mixture, when color is produced, to a pH of about 3.2. The effect of atmospheric oxygen, or other oxidizing conditions, is prevented by maintaining reducing conditions in the reaction mixture through the use of stannous chloride.

Interfering substances in this method are pentoses, pentosans, hexoses, and hexosans. The fermentable hexoses of plant tissues are removed by treatment with yeast. It is not necessary to remove hexoses from animal tissues as they do not exist in quantities that interfere. Glycogen is removed from liver with alcohol. It was not found possible, however, to remove all of the substances which yield furfural under the conditions of this reaction, and the method therefore involves the determination of a correction for the non-ascorbic acid furfural.

Reagents—

Ascorbic acid standards. If ascorbic acid is used as a standard, a solution should be freshly prepared each day. Xylose was adopted as a standard because it keeps indefinitely in saturated benzoic acid solution. A stock solution of xylose in saturated benzoic acid is prepared, 1 cc. of which is equivalent to 0.1 mg. of ascorbic acid. Other standard solutions, containing 0.05, 0.03, 0.02, and 0.01 mg. per cc., are prepared by diluting the stock solution with saturated benzoic acid. Xylose standards must always be checked against pure *l*-ascorbic acid under the conditions of this method, as these two compounds do not have the same rate of furfural formation. The compounds in our hands showed the following relations: 1.23 mg. of xylose dissolved in saturated benzoic acid were found equivalent in furfural-producing capacity to 1 mg. of Merck's pure *l*-ascorbic acid dissolved in either the 17 per cent acetic acid solution used for plant tissues or in the sulfosalicylic-acetic acid mixture used in extracting animal tissues. We, therefore, prepare our stock solution, equivalent to 0.1 mg. of ascorbic acid per cc., by dissolving 123 mg. of xylose in 1 liter of saturated benzoic acid.

Acid-washed norit. Place 100 gm. of norit in a large flask, add 1000 cc. of 10 per cent HCl, and heat to boiling. Filter with the aid of suction and wash with distilled water until the washings give a negative test for chlorides. Remove the charcoal to a vessel suitable for heating. Apply heat slowly at first until the water has evaporated and then heat the material to redness for 15 minutes.

HCl-SnCl₂ solution. To 1 part of H₂O are added 5 parts of concentrated HCl. 4 gm. of SnCl₂ are dissolved in 40 cc. of this solution. This gives a 30 per cent HCl solution containing 10 per cent SnCl₂. It is best to make a fresh solution about once in 2 days. The SnCl₂ in this reagent serves a dual purpose. It reduces reversibly oxidized ascorbic acid and also stabilizes the color produced when aniline is added to furfural.

Alcoholic aniline. The aniline is redistilled and should be colorless when used. To 1 part of aniline 2 parts of 95 per cent ethyl alcohol are added.

Procedure

For Plant Tissues—10 gm. of tissue are thoroughly ground in a mortar with sand under about 50 cc. of 1 per cent oxalic acid. A funnel containing a mat of gauze is placed in a 100 cc. volumetric flask and the ground material is poured upon the gauze. The gauze is squeezed until fairly free of solution and the solid material is returned to the mortar. About 10 cc. of the oxalic acid are added; the mixture is ground again and returned to the gauze filter. The material on the filter is squeezed dry and washed with 1 per cent oxalic acid that has been added to the sand in the mortar to remove material from the latter. This washing is continued until the fluid in the flask reaches the 100 cc. mark. The contents of the flask are mixed thoroughly.

Place 20 cc. of the solution in a small flask and add solid CaCO₃ in excess. When the solution has been neutralized (Congo red paper test), add 1 gm. of Fleischmann's yeast. It is desirable to use a pure strain of yeast, free from vitamin supplements, since the latter increase the difficulty of clarifying with charcoal. Stopper the flask loosely and place in an oven at 38° until the fermentable sugar has been removed. This requires about 1 hour. Remove the mixture to a centrifuge tube and throw down the solids by centrifugation. Place 10 cc. of the centrifugate in a small flask and add 2 cc. of glacial acetic acid. Add approximately 0.5 gm. of acid-washed norit and shake occasionally for 2 minutes. Filter.

(a) **Determination of Total Furfural**—Place 1 cc. of the norit filtrate in a test-tube about 15 × 200 mm. in size. Place in each of three similar tubes 1 cc. of ascorbic acid standard (xylose) solution, the standards used being equivalent to 0.1, 0.05, and

0.03 mg. of ascorbic acid per cc. Add to each tube 1 cc. of HCl-SnCl₂ solution. Place the tubes in a boiling water bath for 15 minutes. Remove and cool. To each tube add 2 cc. of glacial acetic acid, allowing the acid to flow down the sides of the tube in a manner that will remove all water from its sides. Add 3 cc. of alcoholic aniline, letting the mixture flow down the sides of the tube in a manner that will produce a stratification and not permit mixing. Finally, mix the contents of all of the tubes at the same time by shaking thoroughly. Let stand for 10 minutes to permit maximum color development, then read the unknown against the standard which most closely matches it.

(b) *Determination of Non-Ascorbic Acid Furfural*—Place 1 cc. of the norit filtrate obtained above in a 15 × 200 mm. test-tube and in each of three similar tubes place 1 cc. of ascorbic acid standard solution, the standards selected being equivalent to 0.03, 0.02, and 0.01 mg. of ascorbic acid per cc. Add to each tube 1 cc. of 30 per cent HCl. Place the tubes in a boiling water bath for 15 minutes. Remove and cool. Add to each tube 1 cc. of freshly prepared 1 per cent aqueous SnCl₂ solution, then 2 cc. of glacial acetic acid, and 3 cc. of alcoholic aniline. Mix and proceed as in (a) above.

For Vegetable or Fruit Juices—The juice is diluted to 10 volumes with distilled water and, if acid, excess solid CaCO₃ is added. From here the procedure is the same as that followed with the neutralized oxalic acid extract of plant tissues described above. In the case of some fruit juices, such as orange juice, 2 hours fermentation with yeast may be required.

For Animal Tissues—A solution containing 5 per cent sulfosalicylic acid in 10 per cent acetic acid is used to extract animal tissues. The usual procedure is to use 10 cc. of acid mixture for each gm. of tissue. The material is thoroughly ground with sand in a mortar under about 6 volumes of the acid mixture. The extract is filtered through gauze into a volumetric flask and the sand and mortar are washed with the acid solution, the washings being poured through the gauze until 10 volumes of extract are obtained. The extract and washings are thoroughly mixed and the mixture is centrifuged. To 10 cc. of the centrifugate add approximately 0.5 gm. of acid-washed norit, shake occasionally for 2 minutes, and filter. From here the procedure is the same as described for norit filtrates of plant tissues under (a) and (b) above.

For Liver—Liver usually contains enough glycogen to bring about humus formation when the tissue extract is boiled with HCl, a condition which produces an unsatisfactory color when aniline is added. The glycogen is therefore removed with alcohol. The liver tissue is ground under sulfosalicylic-acetic acid mixture and the solution is passed through norit in the usual manner. To 5 cc. of the norit filtrate add an equal volume of absolute ethyl alcohol, mix thoroughly, and centrifuge. From here the procedure is the same as described above for norit filtrates of plant tissues, (a) and (b), except that 2 cc. of the alcoholic centrifugate are placed in the boiling tube instead of 1 cc. of the norit filtrate, and 1 cc. of absolute ethyl alcohol is added to each of the standard tubes containing 1 cc. of ascorbic acid standard solution. The alcohol boils out rapidly when the acid mixture is placed in the water bath, leaving the equivalent of 1 cc. of the original filtrate. The rate of furfural formation is slower while the alcohol is present, however, hence the same amount of alcohol is added to the standards as is present in the unknown tubes.

Calculation—For plant tissues $S/U \times S_1 \times 12 = \text{mg. per gm.}$; for animal tissues $S/U \times S_1 \times 10 = \text{mg. per gm.}$ (S = reading of standard; U = reading of the unknown; S_1 = mg. in standard selected). Determination (a) minus determination (b) = mg. of ascorbic acid per gm. of tissue.

DISCUSSION

The recoveries obtained when ascorbic acid was added to representative tissues are shown in Table I. They are entirely satisfactory. However, recoveries of vitamin added to tissues which contain the vitamin do not reveal how specific the method is. The specificity of this method seems well established by the norit treatment and the determination of the non-ascorbic acid furfural correction. The ease of oxidation of ascorbic acid is well known; and we have no knowledge of any other furfural precursor whose capacity to form furfural is lost by passage through norit and regenerated by reduction with SnCl_2 . These facts seem to indicate that this is an entirely specific method for the determination of ascorbic acid.

It is of distinct advantage that by this procedure ascorbic acid is determined in its reversibly oxidized form as well as in its reduced form. Any step in the analysis which might produce de-

hydroascorbic acid does not influence the recoveries as might be the case with the oxidation-reduction methods.

Oxalic acid is an excellent preservative of ascorbic acid in solution. In experiments with Irish potatoes, turnips, cabbage, spinach, kale, cauliflower, and cow-pea leaves, in which oxalic acid was used as the extractant, it was found that the indophenol-reducing capacity of the extracts did not show appreciable decreases in 2 hours standing. It is suggested that the preservative effects of oxalic acid, when used as an extractant of plant tissues, are due to inhibition of plant oxidases.

TABLE I
Recoveries of Added Ascorbic Acid

Substance	Ascorbic acid present	Ascorbic acid added	Ascorbic acid found	Recovery of added ascorbic acid
	mg. per gm.	mg. per gm.	mg. per gm.	per cent
Tomato juice.....	0.16	1.00	1.10	95
“ “ (autoclaved).....	0.00	1.00	0.99	99
New potatoes.....	0.32	1.00	1.26	95
Turnips.....	0.20	1.00	1.20	100
Spinach.....	0.47	1.00	1.54	104
Kale.....	0.85	1.00	1.78	96
Cabbage.....	0.47	2.00	2.34	94
Red pepper.....	2.20	2.00	4.24	101
Human blood.....	0.01	1.00	1.01	100
Rat liver.....	0.20	1.00	1.26	105
“ muscle.....	0.09	1.00	1.04	95
Sheep adrenal.....	1.18	1.00	2.10	96

The most satisfactory extractant for animal tissues was found to be a mixture of 5 per cent sulfosalicylic acid in 10 per cent acetic acid. The observation of Fujita and Iwatake (1) that metaphosphoric acid is an excellent preservative of ascorbic acid was confirmed, but this acid cannot be used in our procedure as it gives a milky precipitate with aniline.

The oxidation of ascorbic acid by norit is directly related to the amount of norit rather than to the time of standing. It is important, therefore, that an excess of norit be used, otherwise all of the ascorbic acid may not be oxidized, in which case too great a value will be obtained in the correction determination (b). The

amount used in the technique outlined is 10 times that necessary to oxidize the ascorbic acid found in the usual analysis.

With some tissues, *e.g.* spinach, turnips, Irish potatoes, a negative blank results when the filtrate is boiled with HCl, making a correction procedure unnecessary. The correction values seem to be fairly constant in our experience. Thus, a standard correction might be established for tissues in routine work, making a blank determination unnecessary in each analysis. The largest correction values are found in plants which contain pectins. The method is obviously of least value with substances of a relatively high content of non-ascorbic acid furfural precursors. Urine and blood are such substances.

The technique outlined above for the determination of non-ascorbic acid furfural (b) is applicable to the determination of pentoses and pentosans in general. The color obtained remains satisfactory for quantitative comparison for at least 3 hours, a result which is much superior to those obtainable by other procedures in the literature for the determination of furfural by the aniline acetate method. The procedure for determining the non-ascorbic acid correction of plant tissues may be of interest to the plant physiologist as a method for estimating the furfural-producing substances, other than ascorbic acid, in plant tissues.

Comparison with Indophenol Titration Procedure

Of the various methods for the determination of ascorbic acid the Tillmans indophenol titration procedure (2), in modified forms, is the most widely used. It, therefore, seemed of interest to the author to make a study of the Tillmans method, and to compare results obtained by this procedure with those obtained by the furfural colorimetric method, which is based upon entirely different chemical principles.

EXPERIMENTAL

Plant and animal tissues were extracted with 10 volumes of the appropriate acid mixture as described above. After extraction and thorough mixing, an aliquot of the solution was titrated at once with 2,6-dichlorophenol indophenol. A portion of the solution was then treated by the colorimetric procedure. A solution of pure ascorbic acid (Merck's) was prepared each day com-

parative analyses were made, and this solution was used as a standard for the colorimetric method and for standardization of the indophenol. The same tissue extract was thus analyzed by the two methods as nearly simultaneously as possible, each determination being checked against the same standard solution of ascorbic acid.

TABLE II
Ascorbic Acid Content of Plant Tissue

Food	Ascorbic acid		Colorimetric as per cent of indophenol
	Furfural colorimetric method	Indophenol titration method	
	<i>mg. per cc.</i>	<i>mg. per cc.</i>	
Orange juice.....	0.44	0.44	100
Lemon "	0.40	0.43	93
Grapefruit juice.....	0.44	0.47	94
" " canned.....	0.32	0.36	88
Pineapple " "	0.13	0.11	118
	<i>mg. per gm.</i>	<i>mg. per gm.</i>	
Red pepper.....	1.85	1.88	98
Green pepper.....	1.13	1.22	92
Turnip.....	0.20	0.22	91
New Irish potato.....	0.32	0.31	103
Kale.....	0.95	0.99	96
Broccoli.....	0.68	0.71	96
Spinach.....	0.60	0.67	89
Brussels sprouts.....	0.51	0.58	88
Cabbage.....	0.82	0.93	88
"	1.36	1.24	109
Cauliflower.....	0.44	0.45	98
Tomato.....	0.09	0.10	90

Results

The results of these studies are shown in Tables II and III. In Table II a single analysis by each of the methods was carried out upon sixteen representative food substances. The agreement between the two methods is within the limits of experimental error. In Table III a number of analyses by each method were made upon ten animal tissues, the amounts given where more than one analysis was performed being average values. In Table III the two methods are in close agreement in all cases except with liver

tissue. Liver shows a 25 per cent greater value by the indophenol titration method than by the furfural colorimetric procedure, a variation too great to be explained by experimental error.

These results are of considerable interest. As we obtained quantitative recoveries of ascorbic acid added to representative plant and animal tissues by both methods, it follows that these data are accurate. The question that arises is whether these values represent ascorbic acid only. We have presented arguments above to show that the furfural colorimetric method is highly specific. It appears, therefore, that the indophenol titra-

TABLE III
Ascorbic Acid Content of Animal Tissues

Tissue	No. of comparative analyses	Ascorbic acid per gm. tissue		Colorimetric as per cent of indophenol
		Furfural colorimetric method	Indophenol titration method	
		mg.	mg.	
Rat liver.....	6	0.21	0.28	75
“ brain.....	2	0.32	0.32	100
“ intestine.....	2	0.18	0.21	86
“ kidney.....	3	0.17	0.18	94
“ heart.....	1	0.08	0.08	100
“ testis.....	2	0.25	0.28	89
“ spleen.....	1	0.30	0.38	100
Sheep adrenal.....	1	1.18	1.28	92
“ lens.....	2	0.41	0.47	87
“ aqueous humor.....	2	0.13	0.13	100

tion procedure is a specific method for the determination of ascorbic acid in the tissues examined, except liver, since the data obtained by this method are in agreement with those obtained by the furfural colorimetric method.

Our data are not in agreement with certain reports (3-5) in the literature in which it is claimed that ascorbic acid exists in plant and animal tissues in the reversibly oxidized form. Reversibly oxidized as well as reduced ascorbic acid is determined by the furfural colorimetric method. Since the data obtained by this method are in agreement with the indophenol titration procedure, by which ascorbic acid is determined only in its reduced form, it follows that

ascorbic acid was present in the acid extracts of the tissues examined only in the reduced form. In view of our findings it is suggested that the observation of reversibly oxidized ascorbic acid in certain tissues by some authors is a result due to the method of analysis used.

Reports on the alleged existence of reversibly oxidized ascorbic acid in tissues led to the practice of attempting to regenerate the reduced form of the vitamin with H_2S previous to its determination by oxidation-reduction technique. Our data show that with the tissues in Tables II and III such a procedure is superfluous when oxalic, metaphosphoric, and sulfosalicylic acids are used as extractants.

SUMMARY

1. A method for the determination of ascorbic acid as furfural has been developed for plant and animal tissues. The method consists essentially of the determination of the furfural formed by boiling an acid extract of a tissue in which the ascorbic acid has been oxidized by passage through norit, with HCl alone and with HCl containing SnCl_2 . The value obtained with the HCl-SnCl_2 mixture minus that given with HCl alone is the amount of furfural from ascorbic acid. Furfural is determined by the color formed with aniline, stabilized with SnCl_2 and proper amounts of acetic acid.

2. Comparative analyses by the furfural colorimetric and indophenol titration methods have yielded data upon sixteen plant tissues that agree within the limits of experimental error.

3. Close agreement was observed in results obtained by these two methods upon brain, heart, intestine, kidney, testis, spleen, adrenal glands, lens and aqueous humor of the eye. The indophenol titration method gave results upon liver tissue that are 25 per cent higher than those obtained by the furfural colorimetric procedure.

4. The data of this report show that ascorbic acid exists in the reduced form only, in the plant and animal tissues examined.

5. The furfural colorimetric method is of value for its high degree of specificity, for the determination of ascorbic acid in tissues to which the indophenol titration procedure may not be applied, and as a method for determining dehydroascorbic acid.

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STUDIES ON KETOSIS

VIII. QUANTITATIVE STUDIES ON THE OXIDATION OF THE ETHYL ESTERS OF THE FATTY ACIDS*

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Although the theory of β oxidation of the fatty acids has been generally accepted since the classical experiments of Knoop (1) which were further amplified by the work of Embden and Marx (2), Dakin (3), and Ringer (4), the most recent experimental results have been somewhat difficult to interpret on the basis of this theory alone. Thus, Verkade and van der Lee (5) have postulated that ω oxidation is a prominent reaction after trinonylin, tricaprin, and triundecylin, although the dicarboxylic acids so formed are further decomposed either by one-sided or two-sided β oxidation. Jowett and Quastel (6) have suggested from their studies with liver slices that instead of β oxidation a so called "multiple alternate oxidation" occurs as well as α and γ oxidation in certain specific cases.

In earlier studies from this laboratory, Butts, Cutler, Hallman, and Deuel (7) have shown that the conversion of butyric and caproic acids in fasting rats into the acetone bodies by β oxidation is a quantitative one, since identical quantities of the ketone bodies were excreted, as when an equimolecular quantity of sodium acetoacetate was fed. Moreover, no appreciable formation of the ketone bodies followed the administration of the sodium salts of propionic, valeric, heptolic, or pelargonic acid, which would also

* A preliminary report of some of these data has been published (*Proc. Soc. Exp. Biol. and Med.*, **34**, 669 (1936)).

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seem to offer support for the β oxidation theory. However, the acetone body output was found to be approximately double after sodium caprylate than after sodium acetoacetate, which would seem to indicate that there is δ as well as β oxidation under such conditions. A later report (8) from this laboratory has shown that the odd carbon fatty acids (propionic, valeric, heptonic, and pelargonic) are all glycogenic agents, while the corresponding even carbon acids are entirely ineffective in this respect.

Because of the fact that the soaps of the higher fatty acids are somewhat insoluble, no satisfactory proof is available as to the fate of these acids. In our earlier work (7), we were unable to feed sufficient doses of sodium soaps of acids higher than caprylic for quantitative comparisons with the lower members of the series, while Jowett and Quastel found difficulty with decanoic (capric) acid owing to the low solubility of the calcium salt.

However, in the present work we have been able to overcome our previous difficulties by feeding the ethyl esters of the fatty acids. All of these esters up to and including ethyl stearate are liquid at body temperature and may be administered as such. In these studies we have been able to bring forward the first direct proof of the ability of some of the higher fatty acids to be converted into the acetone bodies, as well as to ascertain certain quantitative relations of this change.

EXPERIMENTAL

Male rats from our stock colony 3 to 5 months of age fasted for 24 hours prior to the beginning of the tests were used in the experiments described here. In the main group of experiments the esters were fed by stomach tube in equimolecular quantities of 0.259 mole (equivalent to 15 gm. calculated as acetone) per sq. m. of body surface in two divided doses daily. As in our earlier tests, we have also administered 10 per cent sodium chloride which served to wash the ester out of the stomach tube and to act as a diuretic. The volume of urine amounted to 10 to 20 cc. daily. Urine collections were started daily at 9.00 a.m. Experiments were continued for 4 to 6 days in each case.

In the second group of experiments the esters were usually fed in doses equivalent to 1.88 gm (calculated as acetone) per sq. m. per day. In order to increase the degree of absorption of ethyl

palmitate and ethyl stearate, the ethyl esters were dissolved in Wesson oil. The proportion of oil to ester with the palmitate was an equal amount of each. With the other esters on which simultaneous determinations were made with isomolecular doses of the esters, the proportion of ester to oil was so adjusted that equal amounts of the oil solvent were administered in each case, as well as equimolecular amounts of the ethyl esters.

The ethyl esters employed here were chemically pure products obtained from the Eastman Kodak Company.¹ Their purity was established in each case by the determination of saponification number with standard alcoholic potassium hydroxide as well or by their specific gravity. "Practical" ethyl oleate was used, which gave the theoretical iodine number and saponification value. In all cases the Eastman products were found to give satisfactory agreement with the theoretical value.

The determination of total acetone bodies or of the separate fractions was carried out according to the procedure of Van Slyke (9), while the usual Kjeldahl technique was employed for the determination of the urine nitrogen. The method of Erdmann and Marchand (10) was used for the quantitative determination of mercury in the study of the mercury-acetone precipitates carried out in the latter part of this work.

As was noted before, the Denigès reagent is not specific for acetone but also reacts with valeric acid (7), a result which has been confirmed by Jowett and Quastel (6). In Table I the results of tests made on the Denigès reagent with the various ethyl esters are given.

The reaction of Denigès's reagent with ethyl caproate and the higher esters when added to it in amounts equivalent to the maximum daily doses gave no appreciable precipitates. In most cases it remained as an oily layer on the mercuric sulfate solution during the whole 90 minute period of refluxing. Inasmuch as the acetone body excretion as calculated from the weight of the mercury precipitates after ethyl propionate and ethyl valerate gave practically blank values, these esters were not excreted in sufficient amounts to make the acetone determinations unreliable. The precipitate after the administration of ethyl butyrate was the typi-

¹ One sample of ethyl laurate was kindly furnished us by Dr. S. Lepkovsky of the University of California.

cal yellow one usually obtained with the acetone bodies, so the results are considered reliable on this ester.

Methyl acetoacetate² and ethyl acetoacetate can be hydrolyzed and determined quantitatively as acetone by boiling for 60 minutes with the Denigès reagent without the addition of potassium dichromate by a procedure analogous to that employed by Van Slyke (9) for diacetic acid.

TABLE I

Reaction of Various Ethyl Esters of Fatty Acids with Denigès's Reagent

Ethyl ester used	Procedure 1		Procedure 2	
	Weight of ppt.	Remarks	Weight of ppt.	Remarks
Propionate.....	0.5432	Black ppt.		
Butyrate.....	0.6544	Green "		
Valerate.....	0.0434	" "		
Caproate.....	0.0013			
Heptoate.....			0.0041	
Caprylate.....	0.0000	Only oily layer	0.0004	
Pelargonate.....			0.0000	
Caprate.....	0.0000	" " "	0.0072	
Undecylate.....			0.0000	Only oily layer
Laurate.....	0.0053	" " "	0.0097	
Myristate.....	*0.0140	" " "	0.0186	
Palmitate.....			0.0000	" " "
Stearate.....			0.0000	" " "
Oleate.....			0.0000	" " "

Procedure 1—0.5 cc. of the ester added to copper sulfate and carried through the usual procedure of precipitation with calcium hydroxide and filtration after dilution to 250 cc. 100 cc. of filtrate employed, equivalent to 0.2 cc. of the ester.

Procedure 2—0.2 cc. of the ester added directly to Denigès's reagent, boiled, and filtered.

Because of the large number of experiments carried out here, space does not permit the inclusion of the individual experiments. However, in each series of tests the data have been treated statistically as in our earlier work (11), so that the significance of the average may be ascertained. When the ratio of mean difference

² Kindly furnished us by the National Carbide and Chemical Company.

to probable error of mean difference exceeds 3, the difference is considered a real one.

Results

Experiments with Large Dose of Ester—The results of the acetone body excretion after feeding the ethyl esters with an even number of carbon atoms to fasting rats in doses equivalent to 15 gm. (calculated as acetone) per sq. m. per day are summarized in Table II. Because of irregularities on the 1st day, the results for this day are not considered in the general average. The weighted average is the mean of all tests on each ester from the 2nd to 4th days. The fractionation of the acetone bodies was determined simultaneously on experimental and control rats (the 3rd or 4th day).

The average acetone body excretion after ethyl caproate of 1.95 gm. per sq. m. is almost identical with that of the ethyl acetoacetate controls of 1.91 gm., while the mean of the ethyl butyrate experiments (1.37 gm.) is somewhat lower than the control level (1.74 gm.). On the other hand, the excretion of acetone bodies is more than twice as great after ethyl caprylate, ethyl caprate, ethyl laurate, ethyl myristate, and ethyl oleate, the values of the latter being 7.29, 6.74, 6.75, 5.21, and 5.07 gm. respectively, compared with a control value of 2.01 gm. for the ethyl acetoacetate tests. The average results after ethyl palmitate and ethyl stearate were 2.94 and 2.40 respectively, being only slightly higher than the ethyl acetoacetate controls. It was noted in every instance that an excessive stool formation occurred after these esters were fed, while similar results were not noted in any of the other experiments except to a much smaller amount after ethyl myristate.

The results with the non-ketogenic esters of the fatty acids with an odd number of carbon atoms are recorded in Table III.

The ethyl esters of the odd carbon fatty acids gave entirely negative results on ketonuria in all cases. The average values for propionic, valeric, heptic, pelargonic, and undecylic esters are 0.21, 0.29, 0.22, 0.17, and 0.16 gm. per sq. m. respectively, compared with control values of 0.21 gm. per sq. m. obtained as a mean in 94 experiments.

Experiments on Small Doses of Esters—In view of the fact that the ethyl esters of palmitic and stearic acids were poorly absorbed

TABLE II.

Experiments on Fasting Male Rats Receiving Ethyl Esters of Ketogenic Fatty Acids Orally in Doses Equivalent to 15 Gm. (As Acetone) per Sq. M.

Ethyl ester fed	Body weight	Acetone bodies				Weighted average ^a	Acetone body excretion for 100 gm. rat	Total acetone as diacetic acid	M.d.: p.m.d.† compared with acetate controls	Ratio of acetone body excretion with controls
		1st day	2nd day	3rd day	4th day					
	gm.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	mg.	per cent		
Acetoacetate	212	2.05 (21)	1.72 (21)	2.24 (18)	2.11 (18)	2.01 (57)	40.0	31.1 (12)		
Butyrate	173	0.79 (10)	1.07 (10)	1.55 (9)	1.53 (8)	1.37 (27)	27.3	18.3 (6)		0.80
	178	1.90 (10)	1.30 (10)	1.87 (10)	2.05 (10)	1.74 (30)	34.6	30.5 (6)		
Caproate	220	1.10 (13)	1.66 (13)	2.22 (9)	2.11 (9)	1.95 (31)	38.8	22.3 (7)		1.04
	209	2.00 (12)	1.59 (12)	2.01 (9)	2.25 (9)	1.91 (30)	38.0	30.5 (6)		
Caprylate	195	3.88 (4)	6.56 (4)	7.56 (4)	7.74 (4)	7.29 (12)	145.0	17.4 (4)	14.82	2.65
	199	2.84 (4)	2.78 (4)	3.12 (4)	2.36 (4)	2.75 (12)	54.7	31.4 (4)		
Caprate	211	2.93 (6)	7.38 (6)	6.62 (6)	5.98 (4)	6.74 (16)	134.2	18.6 (4)	10.16	2.83
	206	1.96 (6)	1.98 (6)	2.66 (6)	2.49 (6)	2.38 (18)	47.4	31.8 (4)		
Laurate	215	1.59 (9)	6.20 (9)	9.01 (4)	5.71 (4)	6.75 (17)	134.3	15.7 (4)	9.47	2.83
	237	1.99 (9)	2.12 (9)	2.66 (6)	2.49 (6)	2.38 (21)	47.4	31.4 (4)		
Myristate‡	260	0.57 (4)	3.64 (4)	6.48 (4)	6.43 (1)	5.21 (9)	103.8	17.8 (4)	7.66	2.78
	242	1.65 (4)	1.28 (4)	2.30 (4)	2.02 (4)	1.87 (12)	37.5	32.2 (2)		
Palmitate‡	193	0.49 (4)	1.84 (4)	3.98 (4)	3.05 (2)	2.94 (10)	58.5			1.02
	199	2.86 (4)	2.78 (4)	3.12 (4)	2.70 (4)	2.87 (12)	57.2			
Stearate‡§	246	0.29 (5)	1.57 (4)	2.45 (4)	3.43 (3)	2.40 (11)	47.7	21.6 (1)		1.28
	242	1.65 (4)	1.28 (4)	2.30 (4)	2.02 (4)	1.87 (12)	37.5	32.2 (4)		

TABLE II—*Concluded*

Ethyl ester fed	Body weight	Acetone bodies				Weighted average*	Acetone body excretion for 100 gm. rat	Total acetone as diacetic acid	M.d.: p.a.m.d.† compared with acetoacetate controls	Ratio of acetone body excretion with controls
		1st day	2nd day	3rd day	4th day					
	gm.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	mg.	per cent		
Oleate.....	262	0.55	3.89	5.33	6.28	5.07	101.0	19.4		
		(4)	(4)	(4)	(3)	(11)		(4)		
	242	1.65	1.28	2.30	2.02	1.87	37.5			
		(4)	(4)	(4)	(4)	(12)				

The average acetone body excretion is recorded in bold-faced type for each ester, while the values given below them are for the ethyl acetoacetate controls carried out simultaneously on litter mates. The figures in parentheses represent the number of experiments included in the average.

* 2nd to 4th day only.

† Ratio, mean difference to probable error of mean difference.

‡ Poor absorption of the esters; see text.

§ Half dose only.

|| Wesson oil also added to diet.

when fed in an amount equivalent to 15 gm. per sq. m. (as acetone) and because of the impracticability of intraperitoneal administration, it seemed possible that a quantitative comparison of the acetone body production with the other esters could be made with an intake at a considerably lower level. If more than two acetone body fragments originate from 1 molecule of palmitic or stearic acid, then ketonuria would still be produced after the esters of these acids when the intake of isomolecular amounts of ethyl acetoacetate or even ethyl laurate was insufficient to evoke a ketonuria. Table IV gives a summary of the tests on ethyl stearate.

The excretion of acetone bodies in the urine after the administration of ethyl stearate dissolved in Wesson oil in a dose equivalent to only 1.88 gm. per sq. m. (calculated as acetone) averages 0.99 gm., which is significantly higher than the values obtained after ethyl acetoacetate, ethyl caprylate, ethyl laurate, or ethyl myristate. The experiments on ethyl palmitate and ethyl oleate are given in Table V. Because the results when the ethyl oleate was dissolved in oil are not conclusive from a statistical

standpoint, a second series of tests was made in which the esters were fed in the small dose without the oil.

The mean of 1.16 gm. per sq. m. for the excretion of acetone bodies for the animals receiving ethyl palmitate is significantly higher than the values for the control animals receiving ethyl caprylate or ethyl laurate. In the second group of tests on ethyl oleate, when the dose of the esters was increased to a level equivalent to 4.23 gm. (as acetone) per sq. m., there is a definite ketonuria in the oleate-fed rats which is significantly higher than that for the

TABLE III

Ketonuria of Fasting Male Rats Receiving Sodium Chloride Alone or with Ethyl Esters of Non-Ketogenic Fatty Acids Orally in Doses Equivalent to 15 Gm. per Sq. M.

Substance fed	Body weight	Acetone bodies				Weighted average*	Acetone body excretion per 100 gm. rat
		1st day	2nd day	3rd day	4th day		
		gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	
Ethyl propionate...	231	0.11 (8)	0.26 (8)	0.29 (6)		0.21 (22)	4.2
" valerate.....	245	0.37 (8)	0.27 (9)	0.19 (7)	0.35 (4)	0.29 (28)	5.8
" heptooate....	205	0.18 (6)	0.18 (6)	0.23 (4)	0.30 (4)	0.22 (21)	4.4
" pelargonate..	232	0.11 (3)	0.20 (2)	0.21 (4)		0.17 (9)	3.4
" undecylate...	207	0.13 (11)	0.20 (11)	0.17 (7)	0.14 (5)	0.16 (34)	3.2
Sodium chloride....	226	0.15 (29)	0.20 (29)	0.25 (26)	0.28 (10)	0.21 (94)	4.2

The figures in parentheses represent the number of experiments included in the average.

* Averages of all experiments, 1st to 4th day inclusive.

animals receiving an equivalent amount of ethyl laurate. This would seem to offer cogent evidence that ethyl oleate as well as ethyl palmitate and ethyl stearate gives rise to at least three fragments capable of acetone body formation per molecule.

Analysis of Mercury-Acetone Precipitates—In order to prove that the mercury precipitates formed in the determination of acetone bodies after feeding the various esters were all mercury-acetone compounds, a mercury analysis of various precipitates was made. With pure ethyl acetoacetate the mercury content averaged 76.7 per cent, which agrees well with the mean value of Van Slyke of

76.56 per cent for the chromic acid-free precipitate with acetone. The mercury analyses of the Denigès precipitates with potassium dichromate obtained from the urines of rats which had received the ethyl esters of acetoacetic, caprylic, capric, lauric, myristic, and palmitic acids varied between 75.7 and 77.6 per cent. These are in close agreement with the analysis of Van Slyke of 76.90 per

TABLE IV

Acetone Body Excretion in Urine of Fasting Male Rats Receiving Various Esters in Solution in Wesson Oil in Doses Equivalent to 1.88 Gm. per Sq. M.

Ethyl ester fed	Average weight	Acetone bodies						Weighted average*	Acetone body excretion for 100 gm. rat	M.d.: p.e.m.d.†
		1st day	2nd day	3rd day	4th day	5th day	6th day			
	gm.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	mg.	.
Acetoacetate	220 (15)	0.08 (10)	0.23 (10)	0.40 (10)	0.29 (15)	0.32 (15)	0.39 (5)	0.32 (55)	6.4	8.00
Caprylate	217 (12)	0.25 (10)	0.89 (4)	0.83 (6)	0.51 (12)	0.44 (12)	0.26 (6)	0.56 (40)	11.1	3.98
Laurate	222 (16)	0.15 (16)	0.48 (10)	0.68 (10)	0.44 (16)	0.40 (16)	0.53 (6)	0.49 (58)	9.8	5.24
Myristate	210 (16)	0.19 (16)	0.60 (10)	0.80 (10)	0.61 (15)	0.48 (16)	0.30 (6)	0.57 (57)	11.3	4.48
Stearate	211 (16)	0.32 (15)	0.55 (10)	1.25 (10)	1.01 (16)	0.94 (16)	1.30 (6)	0.99 (58)	19.7	
Wesson oil controls	188 (3)	0.02 (3)	0.04 (3)	0.04 (3)	0.07 (3)	0.14 (3)		0.07 (12)	1.4	

The figures in parentheses represent the number of experiments included in the average.

* 2nd to 6th day only.

† Mean difference to probable error of mean difference, compared with ethyl stearate group.

cent for the Denigès precipitate of β -hydroxybutyric acid and chromic acid and for one of 76.42 per cent when acetone was boiled with chromic acid.

A number of experiments were made with such methyl esters as acetoacetate, butyrate, valerate, caproate, heptate, caprylate, and palmitate. Although the results in general are similar

to those obtained with the ethyl esters, the level of ketonuria was uniformly somewhat lower; moreover, it was invariably noted that

TABLE V

Acetone Body Excretion in Urine of Fasting Male Rats Receiving Ethyl Palmitate or Ethyl Oleate in Doses Equivalent to 1.88 Gm. per Sq. M. Compared with Controls on Ethyl Caprylate or Ethyl Laurate

Ethyl ester fed	Average weight	Acetone bodies						Weighted average	Acetone body excretion for 100 gm. rat	M.d.:p.e.m.d.*
		1st day	2nd day	3rd day	4th day	5th day	6th day			
Fats dissolved in oil										
	gm.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	mg.	
Caprylate	199		0.20 (8)	0.46 (8)	0.55 (8)	1.04 (6)	1.08 (5)			
Laurate	223				0.90 (9)	0.82 (5)		0.68† (49)	13.5	
Palmitate	207		0.58 (9)	0.64 (8)	1.12 (18)	1.50 (15)	1.26 (8)	1.16 (58)	23.1	4.33
Oleate	204		0.27 (9)	0.48 (9)	1.15 (18)	1.23 (13)	1.02 (5)	0.90 (54)	17.9	2.13
Fats administered without oil solvent										
Laurate	221	0.03 (13)	0.09 (13)	0.23† (13)	0.33† (13)	0.44§ (13)	0.62§ (13)		12.3	
Oleate	235	0.03 (13)	0.06 (13)	0.25† (13)	0.41† (13)	0.72§ (13)	1.83§ (13)		36.4	3.76

The figures in parentheses represent the number of experiments included in the average.

* Mean difference to probable error of mean difference, compared with controls.

† Average of all control tests on caprylate and laurate.

‡ 2.82 gm. per sq. m. calculated as acetone.

§ 4.23 gm. per sq. m. calculated as acetone.

|| Average for 6th day only.

toxic symptoms resulted from their continued administration. Experiments on the methyl esters were consequently abandoned and our efforts confined to a study of the ethyl derivatives.

DISCUSSION

In general, the results of the experiments with ethyl esters support the results on the limited series of sodium soaps reported in our earlier work (7). Thus, excretion of acetone bodies after ethyl caproate and ethyl butyrate was practically identical to that found after feeding an equimolecular quantity of the acetoacetate ester. Ethyl caprylate and sodium caprylate behave similarly in bringing about more than twice the ketonuria which occurs when equimolecular quantities of the acetoacetate are fed. Moreover, no appreciable ketonuria follows the administration of the ethyl esters of propionic, valeric, heptonic, pelargonic, or undecylic acids, which is in agreement with our earlier tests on the sodium soaps of the first three acids given above.

The excretion of acetone bodies also was more than twice as great when equimolecular amounts of the ethyl esters of capric, lauric, and myristic acids were fed. One can only interpret such data to mean that more than one fragment capable of transformation to diacetic acid arises from caprylic, capric, lauric, and myristic acids. There is no evidence either in the tests in which a dose equivalent to 15 gm. (calculated as acetone) was fed or in those tests in which a minimum amount of 1.88 gm. of these esters was given that ethyl laurate causes a greater ketonuria than does ethyl caprylate.

When ethyl palmitate and ethyl stearate were fed in the dose equivalent to 15 or to 7.5 gm. (calculated as acetone) per sq. m. per day, the acetonuria averaged less than with the experiments with ethyl caprylate, but far in excess of that of the fasting controls. These results are cogent proof that palmitic and stearic acids are sources of the acetone bodies.

The absorption of these fatty acids was quite low. Although after the 1st day there was practically no formation of stools in the rat when the other esters were fed, a large amount of white, fatty-like stools originated after the palmitate or stearate esters were fed. The stools are first excreted 24 to 36 hours after the first administration of ethyl palmitate or ethyl stearate, in harmony with the results of Cox (12). Although we have not attempted to determine the absorption coefficient of the esters, the value found by Cox after the 2nd day for ethyl stearate in doses comparable with those we employed was only 12 per cent. The

stearate was found to be excreted largely as the free acid and to a less extent as the soaps by this investigator. Only minimum quantities of the unhydrolyzed ester were eliminated.

Had a more complete absorption of these acids occurred with a correspondingly larger amount of them passing through the "metabolic mill," there can be no doubt that far greater amounts of acetone bodies would have been produced and excreted. In order to be able to make better quantitative comparisons, the esters were administered dissolved in Wesson oil in a dose one-eighth that previously employed. Comparisons made with control experiments on the lower esters also dissolved in oil showed that both palmitic and stearic acids possessed a higher ketogenic ability per molecule than acetoacetic, caprylic, or lauric acid. Thus, the average level of ketonuria of 55 experiments in which acetoacetate in oil was administered was 0.32 gm. per sq. m., while that for caprylate was 0.56 (40 tests), for laurate 0.49 (58 tests), for myristate 0.57 (57 tests), and for stearate 0.99 gm. The latter value is significantly higher than for any of the other groups. The mean value for ethyl palmitate when administered at the same level was 1.16 gm. (58 experiments) per sq. m. per day, which is also significantly higher than the caprylate and laurate controls which average 0.68 gm. (47 experiments). Owing to the fact that considerable variation in the threshold of ketosis must exist with different rats, the minimum dose of palmitate or stearate was not always able to provoke a ketonuria. For this reason a considerable variation in individual experiments was noted. However, in order that our interpretation may be an entirely fair one, we have included in our average all experiments carried out, whether a ketonuria resulted or not. Since this average is significantly higher than for the laurate controls, one is forced to the conclusion that palmitic and stearic acids break down into at least three fragments which are convertible to acetone bodies.

Ethyl oleate was well tolerated even when administered in a dose equivalent to 15 gm. per sq. m. per day. More than twice the ketonuria results than occurs with equivalent doses of ethyl acetoacetate. This would seem to be definite proof that oleic acid does not break down at the double bond, according to which well known reaction 1 molecule each of pelargonic and azelaic

acids is formed. This reaction has recently been confirmed by Schoenheimer and Rittenberg *in vitro* (13). If this breakdown of the oleic acid molecule occurs also *in vivo*, no ketonuria should result, in view of the fact that it has been shown that pelargonic acid is not ketogenic and in all probability azelaic acid behaves in a similar manner. The results of Verkade and van der Lee (5) indicate that β oxidation also occurs with the dicarboxylic acids.

TABLE VI

Excretion of Acetone Bodies in Urine after Administration of Ethyl Acetoacetate at Various Levels to Fasting Male Rats Compared with Sodium Acetoacetate

Material fed	Dose per sq.m. per day as acetone	Acetone bodies					Acetone body excretion for 100 gm. rat	Weighted average*
		1st day	2nd day	3rd day	4th day	5th day		
	gm.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	gm. per sq.m.	mg.	gm. per sq.m.
Sodium acetoacetate†	15	3.90 (24)	4.24 (23)	4.83 (20)	5.14 (18)	6.12 (16)	99.5	5.00 (77)
Ethyl acetoacetate	15	2.05 (21)	1.72 (21)	2.24 (18)	2.11 (18)		40.0	2.01 (57)
“ “	3.75	0.40 (4)	0.72 (4)	0.75 (4)	1.08 (4)	1.27 (4)	19.1	0.96 (16)
“ “	1.88	0.06 (4)	0.08 (4)	0.14 (4)	0.19 (4)	0.26 (4)	3.4	0.17 (16)
NaCl controls		0.15 (29)	0.20 (29)	0.25 (26)	0.28 (10)		4.6	0.23 (65)

The figures in parentheses represent the number of experiments included in the average.

* 2nd to 5th day only.

† Grand average from results of Grunewald, Cutler, and Deuel (14).

The greater acetone body formation in rats receiving the small dose of oleate when compared with the laurate controls would seem to indicate that a minimum of three fragments capable of forming acetoacetic acid also originates when 1 molecule of oleic acid is metabolized.

The rat appears to hydrolyze and absorb the ethyl esters satisfactorily. A comparison of the ketonuria obtained by the differ-

ent levels of ethyl acetoacetate with the earlier experiments on sodium acetoacetate and with fasting controls is given in Table VI.

The ketonuria produced as a result of the administration of ethyl acetoacetate is a physiological one similar to that resulting from sodium acetoacetate employed in our earlier studies. Not only is the distribution of the acetone bodies similar to that noted in the fasting ketonuria of the human (70 per cent as β -hydroxybutyric acid), but also the sex difference noted in man and in rats fed sodium acetoacetate is also evident (15). However, the level of ketonuria is considerably lower after the ethyl acetoacetate than after sodium acetoacetate (14). This difference may partly be accounted for by the fact that the stimulating effect on ketosis caused by the alkalosis is avoided, although our previous results indicate that such a stimulatory effect is largely confined to the female (7).

That ethyl acetoacetate as well as the other esters administered becomes part of the "metabolic mill" and is excreted as acetone bodies in amounts which depend on their ketogenic action and also on the ketolytic material available is evident when one considers the result of fractionation of these substances. Ethyl acetoacetate is not excreted as such but only after a large portion has been converted to the β -hydroxybutyric acid. The results of the fractionation tests on the acetone bodies after administration of the other ketogenic ethyl esters also indicate that the β -hydroxybutyric acid is the predominant fraction (80 to 85 per cent of the total).

The increased ketonuria after the higher ethyl ester calculated from the results of the Van Slyke determinations is a real one and not to be traced to reaction of ketones of such higher esters with Denigès's reagent. Dakin (16) showed that, although propyl methyl ketone as well as acetone could be estimated by the iodine titration method, only the latter ketone was precipitated in the gravimetric mercuric sulfate method. Moreover, Jowett and Quastel (6) found that the products of oxidation of caproic, caprylic, and capric acids with liver slices reacted with aniline at identical rates. Since they consider that this would be improbable were the reactants higher homologues of acetoacetic acid, these investigators consider that this is good evidence that acetoacetic acid is the chief β -keto acid produced in the oxidation of the

fatty acids. Much better evidence was given by the fact that identical results on the determination of the acetone bodies were obtained by the manometric and gravimetric procedures employed.

The uniformity in composition of the urine precipitate with Denigès's reagent is substantiated in our own experiments by the analyses of the mercury content on the various precipitates. All the results were close to the values reported by Van Slyke (9) (about 77 per cent). If the higher homologues should have replaced the acetone in the chromic acid precipitate, which Van Slyke considers has approximately the composition $2\text{HgSO}_4 \cdot \text{HgCrO}_4 \cdot 5\text{HgO} \cdot 2(\text{CH}_3)_2\text{CO}$ (actually the CrO_4 replaces 1.09 to 1.23 molecules of SO_4), then the Hg content of the precipitate would have been significantly different. With amyl methyl ketone (from caprylic acid) the mercury would be only 71.4 per cent, while with the ketone from palmitic acid there should have been a value of 64.9 per cent. These results leave no doubt that the Van Slyke procedure in these experiments gives a precipitate only with acetone; it further substantiates the results of Jowett and Quastel that diacetic acid is by far the most important if not the only β -ketone produced by the oxidation of fatty acids having a carbon chain as long as 16 (palmitic acid).

If one considers that the ketonuria which results after the administration of ethyl acetoacetate as merely the result of a temporary overabundance of this component in the tissues, then one might postulate that the higher degree of ketonuria which follows ethyl caprylate and the higher even carbon esters is caused by a greater saturation of diacetic acid in the tissues over short periods of time with a resultingly higher excretion. Although there might conceivably be no difference in the quantity of acetone bodies obtainable from 1 caprylate molecule as compared with 1 acetoacetate molecule, the temporarily higher saturation after the latter would consequently result in a higher *excretion*.

However, although we have as yet no data regarding the rate of absorption of the different esters, it seems highly improbable that the foregoing hypothesis is a valid explanation of the difference. Not only does it seem likely from our present evidence that the rate of absorption of equimolecular amounts of the higher fatty acids is slower (because of the greater bulk due to the higher mo-

molecular weight and because of their lower solubility) but also an increasing length of time should be required to bring about the formation of diacetic acid owing to the time required for the oxidation of the fatty acids by β or other types of oxidation. One can only conclude that the greater ketonuria obtainable after the ingestion of the higher esters is to be accounted for by the fact that quantitatively greater amounts of acetone bodies originate from them than from equimolecular amounts of ethyl acetoacetate.

The experimental evidence recorded here makes it appear unlikely that β oxidation alone is responsible for the degradation of the fatty acids. It seems highly probable that δ oxidation is also a normal procedure by which it is possible to obtain two diacetic acid residues from 1 caprylic acid molecule. The fact that no greater acetone body formation apparently follows the metabolism of ethyl laurate than was noted with ethyl caprylate probably can best be explained by ζ oxidation in the latter case. The two caproate residues so formed are quantitatively converted to acetone bodies, so that ethyl caprylate and laurate would have equal ketogenic values. The results on ethyl palmitate and ethyl stearate are somewhat difficult to interpret because of the complication due to incomplete absorption with the larger doses. However, there seems to be little doubt of their superior value as ketogenic agents to caprylic or lauric acids when fed in smaller amounts. Obviously, at least 3 and possibly 4 acetoacetic acid molecules may originate as a result of successive δ or ζ oxidations.

The greater ketogenic values of caprylic and capric acids are not in harmony with the results of Jowett and Quastel on liver slices. The latter investigators actually note a lower ketogenesis with capric than with acetoacetic acid. We have no explanation for this discrepancy. It is possible that organs other than the liver may be concerned with the metabolism of the fatty acids, so that the tests on liver slices would not give the whole picture. There can be no doubt from earlier results from this laboratory that the ovaries (14) as well as the pituitary gland (17) alter the extent of ketonuria in the intact animal.

On the other hand, it seems likely that the short chain fatty acids with an even number of carbon atoms (butyric, caproic) are quantitatively convertible to the acetone bodies. Not only does

this seem proved from our earlier work (7) but the experiments reported here further confirm this supposition. Ethyl caproate gives an identical value for acetone bodies, as was noted in the control tests with acetoacetate. Although this acetonuria is somewhat lower with ethyl butyrate, this may be partially explained by the toxicity of the latter compound. Also, similar distribution of the acetone bodies on fractionation has been noted in our earlier tests as well as in our present experiments with the butyrate and caproate. There would seem to be no reason for excluding butyric acid from the pathway of oxidation of caproic acid, as Jowett and Quastel postulate in their tests.

There is practically no evidence from our experiments that the fatty acids with an uneven number of carbon atoms are ketogenic. The slight ketonuria with the ethyl esters tested—propionate, valerate, heptate, pelargonate, and undecylate—is no more than was obtained in the control tests with sodium chloride. Although we noted a slight ketonuria after sodium valerate in our earlier tests (7), the results were practically negative after the propionate, heptate, and pelargonate. Moreover, we have shown that these fatty acids are glycogen formers, whereas the even chain carbon fatty acids are not. Although Jowett and Quastel believe that a small but definite ketone body formation can be traced to valeric, heptic, and pelargonic acids (possibly from the acetic acid originating by β oxidation), our results on the whole animal do not support this conception.

The best explanation for our results would be a combination of the "multiple alternate oxidation" theory of Jowett and Quastel with the β oxidation theory as applied to the caproic and butyric acids, as well as to the odd carbon series. With δ oxidation of the latter acids, acetoacetic or butyric acid must originate, in which case a ketonuria should be observed which we have not noted.

SUMMARY

The administration of ethyl acetoacetate, ethyl butyrate, or ethyl caproate to fasting rats results in a uniform ketonuria which is somewhat lower than that produced by their sodium salts. More than twice the ketonuria was observed after the administra-

tion of the ethyl esters of caprylic, capric, lauric, and myristic acids as was found in the acetoacetate controls. This indicates that two fragments capable of forming acetone bodies are produced per molecule of fatty acid.

When ethyl palmitate or ethyl stearate was administered in oil in one-eighth the dose employed in the above tests, or ethyl oleate without oil in a somewhat larger dose, the excretion of acetone bodies was greater than with the laurate or caprylate controls.

One must postulate that palmitic, stearic, and oleic acids break up into at least three fragments per molecule which are capable of transformation to acetone bodies. No appreciable ketonuria followed the administration of the ethyl esters of propionic, valeric, heptioic, pelargonic, or undecylic acid.

It seems probable that caproic and butyric acids as well as the odd chain carbon acids break down chiefly by β oxidation. The even chain carbon acids containing 8 to 14 carbon atoms are probably also broken down by δ and ζ oxidation by a procedure of "multiple alternate oxidation," as suggested by Jowett and Quastel. The fact that ethyl caprate is as good a ketogenic agent as ethyl caprylate would seem to indicate that its chief method of oxidation is to the acetone bodies rather than to the dicarboxylic acids as suggested by Verkade and van der Lee.

The uniformity in mercury content of the precipitates of the urine with Denigès's reagent is evidence that acetone is the only important β -ketone excreted in the urine after the administration of the higher even chain fatty acids.

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THE OXYGEN UPTAKE AND COMPOSITION OF THE SKIN OF RATS IN VITAMIN G DEFICIENCY*

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In vitamin G (B_2) deficiency, in addition to failure of growth, there have been observed loss of hair and dermatitis. This indicated a changed function of the skin. Since several of the vitamins had been shown to be involved in the oxidative processes of the body, it seemed probable that the skin changes in vitamin G deficiency might be associated with an altered oxidative ability of the skin itself.

Investigations of tissue metabolism have revealed a number of oxidation-reduction systems which may be mutually interdependent. The measured oxygen uptake or carbon dioxide production of a cell is the sum total of the changes brought about by all the systems. It is thus impossible at the present time to give the vitamins a definite place in the metabolic systems of the cells. It is significant, however, that vitamins A, B, B_2 (or G), D, and C have been shown to function in some manner in cell oxidations and that a deficiency of these vitamins results in retarded growth, loss of appetite, or lowered metabolism. Vitamin E is known to be involved in fetal development and in normal functioning of the testes, in both of which metabolism proceeds at a high level.

Following the discovery of the flavins, several investigators showed that they could be reversibly oxidized and reduced. From tissue extracts Bierich, Lang, and Rosenbohm (1) isolated a small amount of yellow material which was fluorescent green in the

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oxidized and colorless in the reduced form. They believed the pigment to be identical with Warburg and Christian's (2) yellow oxidation enzyme and with vitamin B₂. Wagner-Jauregg and Ruska (3) and Wagner-Jauregg, Rauhen, and Möller (4) showed that solutions of pure flavins were decolorized by yeast, muscle, or other tissue hash and that the decolorization could be accelerated by lactates, pyruvates, succinates, citrates, and aldehydes. The flavins, however, did not stimulate the oxygen consumption of erythrocytes as did methylene blue. Adler and von Euler (5) showed lacto-, ovo-, and "lumiflavin" capable of increasing the respiration of lactic acid bacteria. In 1933, Kühn, Györgi, and Wagner-Jauregg (6) stated that vitamin B₂ could be separated into two factors, one a skin factor which they called vitamin H. This was confirmed by von Euler and Malmberg (7) and was further clarified by Chick, Copping, and Edgar (8) and by Györgi (9). They showed vitamin B₂ to consist of two fractions, flavin and a pellagra-preventing fraction (vitamin B₆) which is required for the action of flavin in promoting growth, neither flavin nor the supplementary substance alone promoting growth. It is proposed to designate the combination of flavin and vitamin B₆ as vitamin B₂.

The experiments described below are a study of the oxygen uptake and the composition of the skin of white rats in vitamin G deficiency.

Methods

Experimental Animals and Diets—Only male albino rats were used in the determinations. These were secured from the Wistar Institute at weaning time (28 to 30 days old) and they then weighed from 40 to 60 gm.

As soon as received the animals were divided at random into two groups and were placed in separate cages with open screen bottoms. The vitamin B₂-deficient diet of Bourquin and Sherman (10) was used for the production of vitamin B₂ deficiency. Hawk and Bergeim's Food Research Laboratories salt mixture (11) was substituted for the Osborne-Mendel salt mixture.

Preparation of Skin Tissue and Determination of Oxygen Uptake—The determinations of the oxygen uptake were carried out by the method of Warburg (12) in a constant temperature water

bath at $37.5^{\circ} \pm 0.02^{\circ}$ and in a room which was kept between $36-40^{\circ}$.

The animals were stunned by a light blow on the head. The hair of the back was clipped closely with scissors and then shaved after wetting with soft soap solution. Sections of skin were then removed in a saddle shape from the back just below the scapula. The section from the right side was used for the determination of the oxygen uptake, the section from the middle just above the spine was used for the determination of the water and dry matter, and the section from the left side for the determination of total lipid and phospholipid.

The section for the determination of oxygen uptake was placed between pieces of paraffin-beeswax and cut into slices 0.3 mm. or less in thickness. The slices were immediately placed in a warm Ringer-glucose solution in a covered Petri dish. From the Petri dish 50 to 100 mg. of tissue were removed to the previously prepared respiration bottles.

The amount of fat-free dry matter and the phospholipid in the skin sections placed in the manometer vessels were obtained from the value of total lipid and water determined separately. The oxygen uptake or QO_2 (c.mm. of oxygen per mg. per hour at 760 mm. of Hg and 0°) has been calculated from a 2 hour observation period of the uptake in the Warburg respirometers, on the basis of the amount of dry matter, of fat-free dry matter, and also per mg. of phospholipid in the sections of skin. Because of wide variations in the fat content of the skin, it is believed that the oxygen consumption per unit of fat-free dry matter serves as the best basis of comparison of the respiratory rate of normal and deficient skin.

Determination of Water and Dry Matter—The water content was determined on 0.4 to 1.5 gm. of thinly sliced skin by drying at 100° for 2 hours.

Determination of Total Lipid and Phospholipid—Skin sections were cut into thin pieces and the total lipid extracted by the method of Bloor (13). The dried, fatty residue was then extracted with dry petroleum ether, and this extract was made up to a total volume of 50 cc. Of this a 25 cc. aliquot was used for the determination of phospholipid and a 20 cc. aliquot was evaporated for the determination of total lipid. The phospholipid

was precipitated with acetone and magnesium chloride by the method of Bloor (13). Before decantation of the acetone the mixture was left in a refrigerator overnight for the phospholipid to precipitate completely. The phosphorus was determined in the precipitate by the method of Fiske and Subbarow (14). If the molecular weight of lecithin is taken as 777.53 and the atomic weight of phosphorus as 31.03, then it is evident that 1 mg. of phosphorus is equivalent to 25.06 mg. of lecithin. The results are reported as lecithin, although it is realized that the material isolated by this method is a mixture of lipids, chief of which are lecithin and cephalin.

DISCUSSION

In Table I are presented the oxygen uptake, the dry matter, water, total lipid, and phospholipid contents of the skins of twenty-eight vitamin B₂-deficient rats and fourteen normal rats of the same ages. Invariably determinations were made on two deficient and one normal rat at the same time.

Effect of Age—The data presented for the oxygen uptake compared favorably with the observations of Loebel (15) who found for the skin of newborn white mice a QO_2 as high as 5.8 per mg. of dry matter, and for the skin of one newborn white rat a QO_2 of 3.5. The value reported here for mature rat skin is much lower than that found by Klopstock (16) for fresh, human skin (adult). He found an average QO_2 of 2.1 with values ranging from 1.2 to 5.1.

Hawkins (17) states that the metabolism (oxygen uptake) of isolated tissue (liver) of rats is similar at different ages except in the very young. Meyer, McTiernan, and Aub (18) find that in mice age has little effect on the metabolism of isolated tissue. The present investigation confirms their general conclusion in finding that in very young rats the QO_2 may be as high as about 5.0, the value dropping at 4 to 6 weeks to a relatively constant value of about 0.5. From this age on, however, the QO_2 may vary in non-uniform animals (*i.e.* in animals differing in parentage, diet, size, vigor, etc.) from 0.25 to as much as 1.0.

Effect of Vitamin—The rats on the vitamin G-deficient diet up to 24 days showed no striking symptoms other than subnormal growth. After 35 days on the diet all the rats showed a general-

ized thinning of the hair. Several showed extensive development of alopecia on the chest and legs. In every instance the skin of the deficient rats was much thinner than that of the normal rats. In a few instances the skin seemed extremely tender, a slight hyperemia ensuing on shaving.

It is striking that, in view of the claim made by other investigators that the symptoms of vitamin G deficiency are caused in part by inanition, the oxygen uptake and the development of symptoms are not related to changes in weight.

Reference to Table I shows the QO_2 per mg. of water-free and per mg. of fat-free dry matter. At no time does the oxygen uptake of the normal rats fall as low as that of the deficient rats. The basal metabolism of rats declines with age as it does in human beings (19, 20).

The QO_2 has also been calculated per mg. of phospholipid in the tissue used for the measurement of the oxygen uptake. The phospholipid in the sample of respiring tissue was calculated from a determination on a separate sample of tissue. The purpose of the calculation of oxygen uptake on this basis was an attempt to provide some means of measuring the amount of the really living, actively respiring, portions of the tissue, perhaps in this way ruling out the "dilution" effect of increased connective tissue and lipids of the normal and deficient skins. Because of the striking difference in the thickness of the skins of the normal and deficient animals, it was believed possible that the actively respiring tissue might be the same in both animals. This is in accordance with the suggestion of Bloor (21) who found that beef muscles, as well as the muscles of smaller animals and birds, when arranged in the order of their decreasing phospholipid contents, are also the decreasing order of their physiological activity. Assuming this to be generally true, it was thought that the amount of phospholipid in the respiring tissue might serve as a better index of the quantity of active cell layers than the fat-free dry matter. Kooyman (22) has reported the basal cell layers of human skin to contain more phospholipid than the layers above or below.

It is evident from Table I that the oxygen uptake in relation to phospholipid is extremely irregular in the deficient animals but fairly regular in the normal animals. In spite of the variability of the phospholipid-oxygen uptake of the normal and deficient

TABLE I
Deficient and Normal Rats in Order of Days on Diet

Animal No.	QO ₂ , c.mm. per hr.		Dry matter		Water content of fresh		Total lipid		Phospholipid (P × 25.06)				Change in weight from initial		Total age	Days on diet	Weight	
	Per mg. dry mat-ter	Per mg. fat-free dry matter	Per mg. phospho-lipid	H ₂ O-free, of fresh weight	Fat-free, of fresh weight	Fresh weight	H ₂ O-free dry matter	Fresh weight	H ₂ O-free dry matter	Total lipid	Fat-free dry matter	Initial	Final					
Deficient animals																		
23-A	1.97	4.60	67.60	30.00	11.11	70.00	17.19	57.31	0.88	2.92	5.10	6.85	-2.7	36	5	55.0	53.5	
23-B	1.51	2.26	48.45	29.60	19.64	70.41	9.95	33.63	0.93	3.12	9.29	4.71	-2.2	36	5	67.0	65.5	
22-D	1.95	3.07	64.65	31.10	19.81	68.90	11.28	36.29	0.94	3.03	8.36	4.76	+2.7	37	6	55.0	56.5	
22-E	3.80	2.89	62.35	30.72	18.94	69.28	11.61	38.01	0.89	2.90	7.67	4.69	+1.0	37	6	54.0	54.5	
21-L	1.46	2.38	61.2	36.07	22.18	63.93	13.88	38.50	0.87	2.41	6.26	3.92	-7.4	44	13	54.0	50.0	
21-K	1.38	2.79	53.35	35.63	17.57	64.37	18.05	50.67	0.93	2.61	5.17	5.31	+1.7	44	13	59.0	60.0	
14-B	0.72	1.40	51.95	38.99	19.88	61.01	19.01	49.01	0.54	1.39	2.84	2.73	+36.0	50	22	50.0	68.0	
14-C	0.70	1.25	45.1	40.04	22.14	59.96	17.90	44.12	0.64	1.57	3.55	2.87	+38.5	50	22	57.0	79.0	
15-A	0.98	1.11	57.85	30.53	26.67	69.47	3.83	12.56	0.54	1.77	14.16	2.03	+10.0	52	24	49.0	54.0	
15-B	0.675	0.80	41.8	34.18	28.35	65.82	5.82	17.05	0.58	1.68	9.94	2.04	+7.3	52	24	54.5	58.5	
9-B	0.61	0.65	36.4	32.10	30.29	67.90	1.81	5.60	0.55	1.71	30.55	1.82	+15.0	63	35	63.5	73.0	
9-C	0.95	1.06	56.15	32.80	29.30	67.20	3.50	10.68	0.56	1.69	15.85	1.89	+24.0	63	35	37.5	46.5	
7-D	0.53	0.56	26.6	30.34	28.35	69.66	1.94	6.41	0.63	2.06	32.22	2.20	-20.0	63	36	50.0	40.0	
7-N	0.74	0.77	53.4	32.98	30.73	67.02	2.27	6.88	0.47	1.43	20.80	1.53	-2.0	63	36	51.0	50.0	
11-C	0.72	0.77	46.0	31.54	29.55	68.46	1.99	6.31	0.50	1.58	25.00	1.68	+26.0	67	39	62.0	78.0	
11-D	0.74	1.15	41.45	34.80	22.44	65.20	12.36	35.52	0.64	1.82	5.14	2.83	+35.5	67	39	45.0	61.0	
2-8	0.48	0.53	38.05	35.03	33.26	64.97	1.73	4.94	0.50	1.42	28.78	1.49	+15.0	79	49	56.5	65.0	
2-T	0.94	1.005	62.85	32.42	30.52	67.58	1.86	5.76	0.54	1.67	29.06	1.77	-1.0	79	49	49.0	48.5	

17-B	0.63	1.02	50.9	41.31	25.55	58.69	15.76	38.16	0.52	1.25	3.27	2.02	+45.5	79	51	57.0	83.0
17-A	0.62	1.21	40.85	38.11	17.62	61.89	20.49	53.76	0.53	1.38	2.56	2.98	+46.0	79	51	52.0	76.0
4-A	0.368	0.383	23.05	31.90	30.60	68.10	1.30	4.08	0.51	1.58	38.85	1.65	+48.0	85	55	41.5	61.5
4-B	0.184	0.205	12.1	34.34	23.56	65.66	1.45	5.82	0.41	1.64	28.23	1.74	+45.0	85	55	42.0	61.0
13-B	0.51	0.71	26.7	33.07	23.81	66.93	9.26	28.01	0.64	1.94	6.94	2.70	+28.0	83	55	62.5	80.0
13-C	0.64	1.09	48.35	40.52	23.89	59.48	16.63	41.05	0.54	1.33	3.25	2.26	+51.0	83	55	55.5	83.5
1-A	0.68	0.79	48.95	35.21	30.08	64.79	5.12	14.56	0.50	1.42	9.75	1.66	+40.0	86	56	54.5	76.5
1-B	0.47	0.5	20.6	33.63	31.83	66.37	1.79	5.34	0.79	2.36	44.23	2.49	+37.5	86	56	40.0	55.0
10-M	0.63	1.27	36.25	39.41	19.85	60.59	19.42	49.44	0.69	1.76	3.56	3.49	+83.0	106	79	44.5	81.5
10-H	0.47	0.92	21.75	38.00	16.37	62.00	15.48	48.60	0.63	1.98	4.08	3.86	+58.0	106	79	67.0	106.0

Normal animals (controls)

24-C	2.50	3.60	97.7	31.47	20.86	68.53	10.60	33.71	0.81	2.56	7.61	3.86	+7.1	36	5	63.5	68.0
24-F	1.87	3.29	74.7	35.18	19.89	64.82	15.28	43.44	0.88	2.50	5.76	4.42	+2.0	37	6	56.0	67.0
24-H	2.00	3.23	83.3	32.93	20.75	67.07	12.17	36.96	0.80	2.41	6.53	3.83	+33.0	44	13	60.0	80.0
6-M	0.97	1.92	57.3	44.87	22.74	55.13	22.12	49.31	0.79	1.76	3.58	3.49	+87.7	49	22	57.0	107.0
18-A	1.13	2.00	80.8	39.31	60.69	60.69	17.08	43.45	0.57	1.44	3.33	2.55	+80.0	52	24	50.0	90.0
12-A	1.03	1.43	114.9	37.53	27.05	62.47	10.48	27.92	0.34	0.91	3.23	1.25	+84.5	63	35	59.0	109.0
12-B	0.79	1.49	67.9	43.92	23.16	56.08	20.76	47.26	0.52	1.17	2.48	2.22	+136.0	67	39	44.0	104.0
5-R	1.11	1.75	99.6	40.90	25.60	59.10	15.30	37.41	0.48	1.16	1.85	3.10	+161.0	79	49	43.5	113.5
16-A	1.02	1.54	94.5	39.60	26.01	60.36	13.63	34.38	0.53	1.34	3.97	2.05	+121.0	79	51	58.0	128.0
16-B	0.87	1.85	72.3	43.80	22.09	56.20	21.72	49.58	0.57	1.29	2.61	2.55	+155.0	80	52	45.0	115.0
5-S	0.87	1.28	66.6	39.64	26.70	60.36	12.94	32.64	0.52	1.35	4.04	1.96	+190.0	85	55	42.0	122.0
6-C	1.05	1.41	87.7	38.57	28.63	61.25	9.93	25.67	0.46	1.19	4.62	1.60	+200.0	86	56	43.0	123.0
12-C	0.56	1.26	56.1	39.98	17.80	60.02	22.18	55.48	0.40	1.00	1.81	2.26	+195.0	89	61	46.0	136.0
6-A	1.03	1.49	76.4	40.27	27.84	59.73	12.43	30.88	0.55	1.35	4.39	1.96	+172.5	105	75	58.0	158.0
Average of normal rats.....			39.14	23.67		60.84			0.59	1.53		2.65					
Average of deficient rats.....			34.15	24.42		64.13			0.64	1.90		2.84					

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rats the difference between the two series indicates a higher level of activity in the normal than in the deficient rats.

	Phospholipid content		
	Fresh skin	Water-free, dry matter	Fat-free, dry matter
	per cent	per cent	per cent
Average normal.....	0.585	1.53	2.65
“ deficient.....	0.638	1.90	2.84

As calculated on the three different units the per cent of phospholipid is somewhat higher in the deficient rats than in the normal. If the phospholipid content is to be accepted as a measure of the relative proportion of respiring protoplasm, it appears from the above summary that the skin of deficient rats contains proportionally more active living matter than the skin of normal rats. This might be expected from the thinness of the skin of vitamin B₂-deficient rats in which fat and connective tissue seem reduced. If the rate of respiration depends only on this living matter, it would be expected that the respiration per unit weight of skin would be greater in the deficient rats than in the controls, assuming that the living matter of each respired at the same absolute rate. Since the oxygen uptake per mg. of fat-free dry matter and in relation to phospholipid is lower in the deficient rats, it means a much greater depression of the real respiration than the figures indicate.

But it may be that the phospholipid content of protoplasm does not remain altogether constant. Several investigators (quoted from the review of Sinclair (23)) have reported for vitamin B₁-deficient rats, rabbits, and pigeons a decrease of 18 to 40 per cent in the phospholipid of the brain, liver, kidney, and lungs, but no change in the muscles and skin. In rats deprived of either vitamin B₁ or A the liver phospholipid is one-half to one-third of normal. Remp and Bing (24) believe inanition to be a feature of the development of vitamin G deficiency. They conclude that in mice, both calorie-deficient and vitamin G-deficient, the total body phospholipid is the same (expressed as mg. in the total body). However, when calculated as per cent of the net body weight (author's calculations from the data of Remp and Bing), it appears that there is actually an increase. Normal, calorie-deficient,

and vitamin G-deficient mice show respectively 1.10, 1.60, and 1.85 per cent phospholipid of the net body weight. The increased percentage of body phospholipid in mice on the calorie-deficient and vitamin G-deficient diets may be looked upon, therefore, as a conservation of phospholipid, other constituents of the body having been called upon as sources of energy, the phospholipid as an essential constituent of all living matter remaining unchanged. Stored fat, carbohydrate, and some protein were consumed.

It is concluded, therefore, that there is a marked reduction in the rate of respiration of the skin cells of rats on the vitamin G-deficient diet.

To prove that vitamin G was responsible for this reduction experiments were tried of adding flavin to the rat skin *in vitro* to see if it would increase the respiration.

Effect of Flavin in Vitro on Oxygen Uptake of Skin of Normal and Deficient Rats—A sample of flavin was prepared, by the method of Booher (25), from powdered whey. There was obtained a small quantity of red wax-like material, which, when dissolved in water, was yellow with a strong green fluorescence. This material corresponded to the properties described by Kuhn for lacto-flavin (26) in that in water solution it was yellow with a strong green fluorescence, it was decolorized by zinc and bisulfite in acid solution, the color returning when the fluid was decanted from the zinc powder and shaken with air. It was soluble in chloroform and insoluble in ethyl ether.

This material was dissolved in water and the solution used in the preparation of Ringer-glucose solution. The final solution as used with the tissue for the determination of the oxygen uptake was still of such concentration that it was quite yellow and still retained the strong green fluorescence.

The effect of this solution on the oxygen uptake of several rats is summarized below as QO_2 per mg. of water-free, dry matter.

	Without flavin	With flavin
Normal.....	2.03	1.88
Deficient.....	1.26	1.21
Normal.....	1.73	1.68
Deficient.....	1.12	1.13

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The small differences observed are negligible, since they fall well within the experimental error. The failure to stimulate the oxygen uptake is not surprising in view of the observations cited previously, that flavin alone does not function as vitamin G and that the flavin requires for its action as vitamin G an additional substance, the nature of which is unknown (27). If further investigation confirms the finding that vitamin G is a complex consisting of flavin plus a supplementary substance (vitamin B₆), and that neither of these alone is sufficient to prevent pellagra (8, 9), then the above observation that flavin does not stimulate the oxygen consumption of the skin of the vitamin G-deficient rat may be interpreted to mean that the supplementary substance was lacking.

Other Changes in Skin of Rats in Vitamin G Deficiency—Taking the averages of all the animals in the two groups, including those at the beginning which had been on the diet only a short time, we have the following comparison, all as per cent of the fresh tissue.

	Water-free dry matter	Fat-free dry matter	Water
Normal.....	39.14	23.67	60.84
Deficient.....	34.15	24.42	64.13

The differences between the normal and deficient rats is great enough to indicate that in the deficient rats there is a slightly increased fat-free dry matter content, a somewhat greater decrease in water-free dry matter, and the same degree of increase in water content. It appears, therefore, that the development of connective tissue is not impaired in the skin of deficient rats. The greatest effect is in the content of neutral fat (as has been shown by von Euler and Malmberg (7)) which is in sharp contrast to the relative constancy of the phospholipid content.

SUMMARY

The oxygen consumption of the skin of vitamin G-deficient rats declines to a much lower level than in normal rats at the same age. The difference is not related to change in weight and hence is not caused by inanition. The oxygen uptake per mg. of phospholipid of the skin also declines markedly in the deficient

rats. This indicates a lowering of vital activity in the skin of these animals.

In vitamin G deficiency the total fat content is markedly decreased, while the phospholipid content shows only a slight increase.

I am indebted to Dr. A. P. Mathews for helpful criticism and guidance in the course of this study.

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A SIMPLIFIED METHOD FOR THE PREPARATION OF CRYSTALLINE PROGESTERONE* FROM PIG OVARIES

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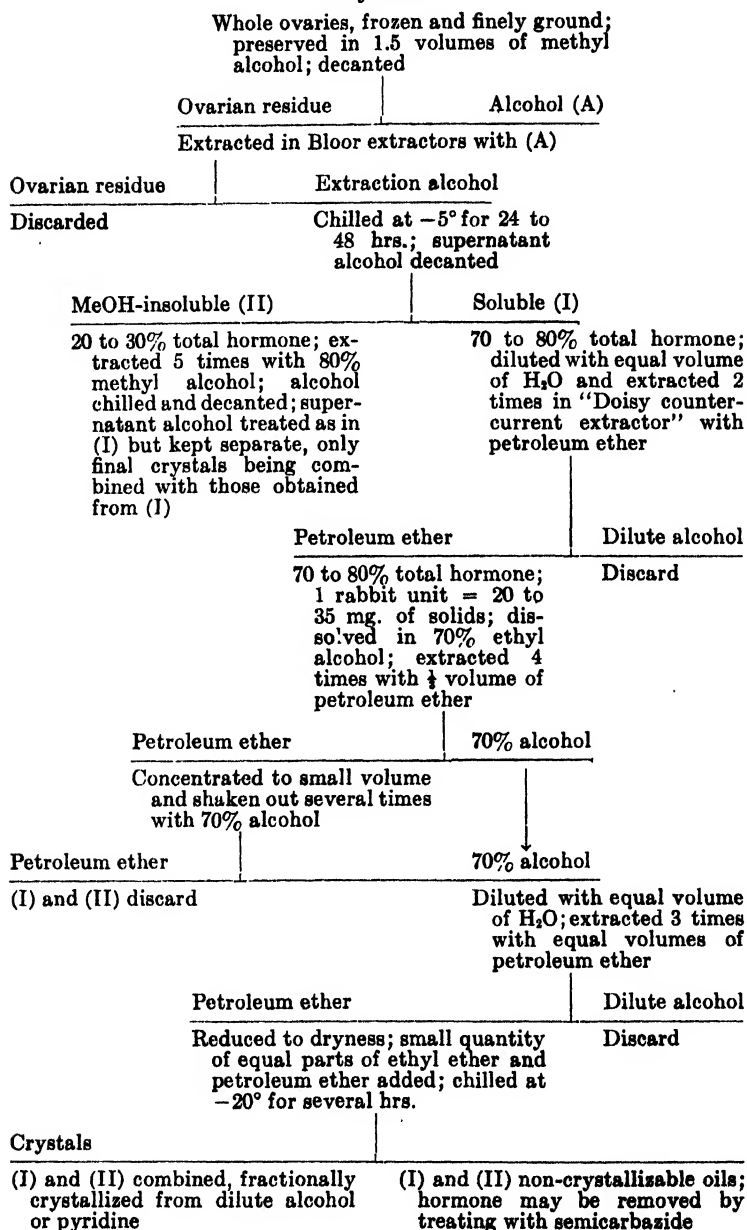
With the advent of crystalline progesterone in 1934 (Slotta, Ruschig, and Fels; Butenandt, Westphal, and Hohlweg; Hartmann and Wettstein; Wintersteiner and Allen), the need for greater amounts of the hormone for physiological purposes became pressing. We were prompted, therefore, to seek a more rapid and less expensive method of obtaining large quantities of relatively pure material from which the crystalline product could be obtained. The method which we are describing in this paper is based upon experience gained in the processing of two large batches of pig ovaries, one of 200 pounds and the other of 500 pounds, each of which was so subdivided that the method has been followed four times. Fresh ovaries were collected and sorted at the abattoir in Chicago, only those containing large pink corpora being retained. They were frozen individually at -17.8° within a few hours after slaughter, and subsequently shipped in a refrigerator car to the laboratory where they arrived in good condition. The tissue was passed through a power meat grinder while still frozen and preserved in 1.5 volumes of absolute methyl alcohol.¹ After remain-

* The name progesterone, proposed by Allen, Butenandt, Corner, and Slotta (1935) for the progestational hormone of the corpus luteum in pure form, has been accepted by the Council on Pharmacy and Chemistry of the American Medical Association (*J. Am. Med. Assn.*, 106, 1808 (1936)).

¹ A grade equal to that of Commercial Solvents Corporation methanol should be used to avoid introduction of contaminating products which interfere with subsequent steps in the procedure.

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Diagram 1



ing in the preserving alcohol for 48 hours or more the minced tissue is filtered through gauze bags as needed, placed in 1 pound lots in Bloor extractors, and extracted as in the older method (Allen, 1932).

The method to this point differs from the earlier one essentially in that methyl alcohol is used as a preserving and extracting agent rather than ethyl alcohol. Methyl alcohol is a poorer solvent for fats than ethyl alcohol, consequently if it extracts the hormone satisfactorily it would be preferable to ethyl alcohol, since the crude extracts would contain less impurity. Actually this is so. We have found that three extractions with boiling methyl alcohol in Bloor extractors remove practically all of the hormone from the minced tissue and at the same time leave considerable fatty material behind.

The extraction alcohol from the preceding step is chilled at -5° for 24 hours or more, thus precipitating a large amount of fat. This precipitate is processed separately (MeOH-insoluble (II), Diagram 1). The supernatant solution (approximately 60 to 70 per cent alcohol (I)) is diluted with an equal volume of water, and then passed through a continuous counter-current extractor against petroleum ether.² (The solution becomes turbid on dilution and remains so even after being extracted with petroleum ether.) This method of removing the progesterin from the diluted alcohol is the new part of the present procedure. With the older method large volumes of alcohol had to be concentrated, aqueous phases extracted with ether, phospholipids removed by precipitation with acetone—all time-consuming and often troublesome processes—but with the new procedure these steps are all eliminated and replaced by a process which is semiautomatic and which yields a comparatively pure product.

The extractor (Fig. 1) used in this step is a modification of one designed by Veler, Thayer, and Doisy (1930) for the extraction of estrin from human pregnancy urine. The petroleum ether boils in flask *A* and is condensed in *B* from which it passes through *C* to the bottom of the baffle column *D*. It rises in this column to the upper separator *E* and thence overflows to *A*. The dilute alcohol solution is introduced into *E* from the Mariotte bottle (McCarthy,

² B.p. range $55-65^{\circ}$. Purified over H_2SO_4 , washed with NaOH, and redistilled.

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1934) *F* and flows downward, against the rising current of petroleum ether in the baffle tube, separates in the lower separator *G*, rises in the tube *H*, and overflows through the adjustable tube *I*. *J* is a U-tube manometer which measures the pressure of the alcohol as it is introduced into *E*. This is calibrated to serve as a flowmeter. *K* is a bottle and jet from which slowly drips absolute methyl alcohol. This dripping alcohol is necessary to disperse

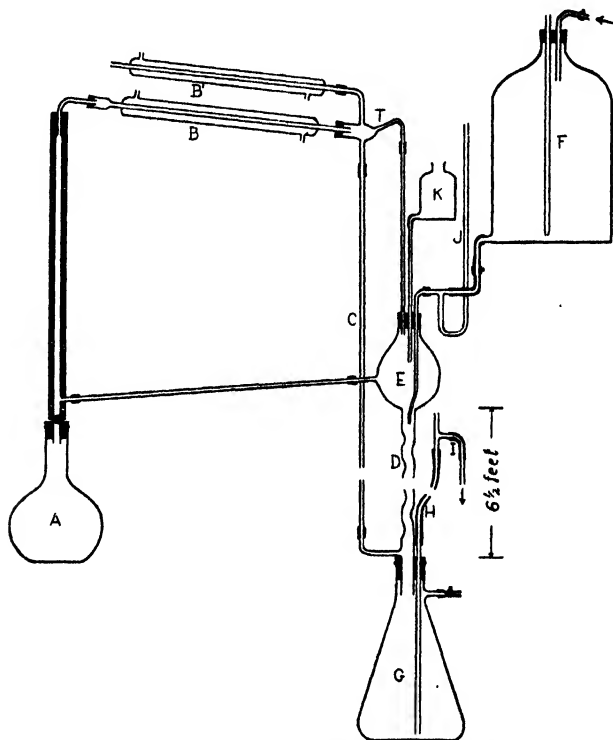


FIG. 1. Extractor used in the preparation of crystalline progesterone

any viscous emulsion that is carried up into *E* where it may flow back into *A*. This slow drip is completely effective. *B'* is a second condenser opening to the atmosphere and so connected to the first condenser and the flask *E* that no petroleum ether is lost. This provides such good condensation that the apparatus can be run for days even in summer without any petroleum ether being added, but it is our practise to change the petroleum ether after 24 hours.

The machine as set up in our laboratory circulates the petroleum ether at a rate of about 30 cc. per minute. The alcohol is run through at the rate of 90 to 110 cc. per minute. When operated at this speed, about 50 liters can be extracted once in an 8 hour day. The apparatus requires very little attention and in actual operation the only adjustments necessary to maintain continuous operation are to keep the alcohol and the petroleum ether entering the column at a constant rate. The alcohol is controlled by using a Mariotte bottle to maintain a constant pressure at the drain regardless of the amount of alcohol in the bottle and the use of an adjustable clamp. The overflow *I* may occasionally have to be

TABLE I

Yields of Progesterin Obtained by Use of "Doisy Counter-Current Extractor"

Fraction	Preparation 113-A		Preparation 113-B		Preparation 114-A		Preparation 114-B	
	Yield per kilo	Solids per rabbit unit	Yield per kilo	Solids per rabbit unit	Yield per kilo	Solids per rabbit unit	Yield per kilo	Solids per rabbit unit
	rabbit units	mg.	rabbit units	mg.	rabbit units	mg.	rabbit units	mg.
1st petroleum	14.7	21	14.7	35.4	14.7	23.4	12.2	19.2
2nd "	4.4	25			4.4	34		
3rd "					0.9	90	0.6	26.4
MeOH-insoluble.....	3.1	2000*	1.8	125	7.3	116	5.9	100
Total yield.....	22.2		16.5		27.3		18.7	

* Phospholipids removed by precipitation with acetone; no other purification carried out.

raised or lowered from time to time to maintain the proper levels in the upper and lower separators *E* and *G*, but in general once the proper level has been found by trial, it does not require changing provided the flow of alcohol is kept constant. The Mariotte bottle, the top of which is near the ceiling, is most easily filled by suction from a water pump by use of a third tube through the stopper (not shown in Fig. 1). For the Mariotte bottle to function as a pressure regulator all openings at the top must be closed except the tube reaching to the bottom of the bottle. The second condenser *B'* is not placed above the first condenser, as illustrated, but back of it. The distance from the trap *T* to the overflow from *E* is 33 inches.

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The efficiency of the column operating under these conditions has been tested by assaying the amount of hormone obtained from each run (Table I). With Preparations 113-A and 114-A, where the first and second extractions were assayed separately, 3 times as much hormone was obtained in the first extraction as in the second. This by itself means that very little would be obtained by a third extraction, and by actual test a third extraction of Preparation 114-A yielded only one-fifth as much as the second. A third extraction, therefore, is unnecessary.

The hormone collecting in the petroleum ether by this process is surprisingly pure, considering that actually only two steps are employed, *i.e.* extraction of the tissue with methyl alcohol and subsequent extraction of the diluted alcoholic extract with petroleum ether. The best preparation (No. 114-B) contained 19 mg. of solids per rabbit unit and the poorest 35 mg. This fraction contains about 70 to 80 per cent of the total amount of hormone present; the other 20 to 30 per cent is present in the fat which settles out of the extraction alcohol (MeOH-insoluble (II)) and is worked up separately.

The petroleum ether is distilled off and the resulting oil is dissolved in 70 per cent ethyl alcohol and extracted 5 times with 0.25 volume of petroleum ether. During this step most of the hormone remains in the alcohol, whereas the majority of the solids is transferred to the petroleum ether. The petroleum ether is washed back several times with $\frac{1}{3}$ volumes of 70 per cent alcohol, this being combined with the original. The 70 per cent alcohol is next diluted with an equal volume of H_2O and the solution extracted four times with 0.5 volume of petroleum ether. During this maneuver the hormone is transferred to the petroleum ether phase, and the estrin is retained in the 35 per cent alcohol phase (Allen and Meyer, 1933). The petroleum ether is distilled to dryness and a few cc. of absolute ethyl ether are added. A copious precipitate forms, and by adding about 10 cc. of petroleum ether and chilling at -20° a considerable quantity of precipitate is obtained. These crude crystals are for the most part a mixture of progesterone and 3-hydroxy-20-ketoallopregnane. The mother liquor is a light yellow oil which is still very potent. This can be further purified by distilling in a high vacuum (Wintersteiner and Allen, 1934), but it is somewhat easier to treat it with semicarbazide, thereby precipitating the

progesterone as the disemicarbazone (Butenandt, Westphal, and Hohlweg, 1934) (the semicarbazone of the inactive hydroxy ketone is also obtained).

The semicarbazide reaction is carried out as follows: The oil is dissolved in as small a quantity of 80 per cent ethyl alcohol as possible, and an excess of semicarbazide hydrochloride to which has been added an equivalent of sodium acetate (both dissolved in 80 per cent alcohol) is added. This is refluxed for 45 minutes during which a copious yellowish white precipitate is obtained. After standing overnight at room temperature the precipitate is filtered off, washed repeatedly with absolute alcohol and ether, alcohol, water, and dried. The oils remaining after this treatment are virtually inactive. In one case, for example, 800 mg. of oil obtained after 480 mg. of semicarbazone precipitate had been filtered off contained less than 12.5 rabbit units of hormone.

The recovery of the hormone from the semicarbazone precipitate has not been completely successful. From solubilities and melting points it appears that the precipitate is for the most part progesterone-disemicarbazone, but we have not been able to recover a good yield of this. However, the only crystalline compounds isolated from this precipitate have been α - and β -progesterone together with a very small quantity (10 mg.) of a substance crystallizing in flakes and melting at 65–70°. Little significance is attached to this latter compound at the present time, since it is not pure.

The semicarbazone precipitate has been decomposed by several methods. The first one tried was that employed by Ruzicka and Wettstein (1935)—dissolving in 2 parts of 95 per cent alcohol and 1 part of 70 per cent H_2SO_4 and warming. By use of this method 50 mg. of β -progesterone were recovered from 150 mg. of semicarbazone precipitate. The method of Butenandt, Westphal, and Hohlweg (1934), in which more dilute sulfuric acid was used, proved impractical because the precipitate did not dissolve. The most promising results were obtained when the precipitate was dissolved in equal parts of glacial acetic acid, in which it is very soluble, and 1 N HCl. This mixture was heated on the steam bath for 30 minutes, diluted with an equal volume of water, and extracted repeatedly with ethyl ether. The ether solution was washed several times with small quantities of 0.5 N Na_2CO_3 , until the washings returned alkaline, and then with water until neutral.

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The ether was evaporated, leaving a good yield of slightly yellowish needle-shaped crystals which, it was hoped, would prove to be progesterone. However, after two crystallizations from 80 per cent ethyl alcohol, it was evident that they were not progesterone but probably a monosemicarbazone of progesterone, since the melting point was 205° (with decomposition). Consequently, to effect further decomposition they (approximately 500 mg.) were dissolved in 10 cc. of 60 per cent alcohol, 1 cc. of 15 N HCl was added, and the mixture was refluxed for $\frac{1}{2}$ hour. The alcoholic solution was diluted with 2 volumes of water and extracted 3 times with 100 cc. portions of petroleum ether. The petroleum ether was washed with 0.5 N Na_2CO_3 and water as above and then evaporated to dryness, leaving a pale yellow wax from which 200 mg. of progesterone were isolated by crystallization from dilute pyridine. Neither of these methods of decomposing the semicarbazone precipitate can be considered satisfactory, but in practice more hormone has been recovered by this method from previous non-crystallizable oils than can be obtained by high vacuum distillation.

The recrystallization of the crude crystals obtained either from the concentration of the petroleum ether fraction or that from the decomposed semicarbazone can be carried out from either dilute alcohol or dilute pyridine. Two or three crystallizations from 60 to 70 per cent ethyl alcohol serve to separate most of the hydroxy ketone from the progesterone, but to get pure material fractional crystallization is necessary. Dilute pyridine appears to be preferable to dilute alcohol, since the hormone tends to stay in the α form rather than to give mixtures of α and β as so often happens when dilute alcohol is used.

The procedures described above, the latter steps of which cannot be considered as ideal, have given very good yields of pure material compared to any method hitherto published. It is of interest also that the crude crystals contain much less of the inactive hydroxy ketone when prepared by this method than by the previous one (Wintersteiner and Allen, 1934). From a lot of 1.95 gm. of crude crystals, obtained from 400 pounds of ovaries, we obtained by fractional crystallization 1.03 gm. of practically pure progesterone, and 80 mg. of pure and 180 mg. of nearly pure 3-hydroxy-20-ketoallopregnane. It is probable that other compounds may be present, since a very small amount of material melting at

202–204° was obtained. This yield of progesterone represents 25 per cent of the hormone present in the original lot of tissue.

The fraction insoluble in 70 per cent methyl alcohol (MeOH-insoluble (II)) can be purified by the old method (Allen, 1932) with acetone to precipitate the phospholipids, etc., but we have found it more practical to extract this heavy oil with 80 per cent methyl alcohol. In practice this is carried out as follows: To 1 volume of oil about 4 volumes of boiling absolute methyl alcohol are added; the mixture is thoroughly agitated for several minutes and then allowed to stand for several hours during which time the oil collects on the bottom. Enough water is then added to dilute the alcohol to 80 per cent. This gives considerable additional precipitate which settles out better if the solution is chilled at -5° for several hours. Finally the clear mother liquor is decanted and the entire procedure repeated five times. The combined 70 per cent methyl alcohol fractions are diluted with an equal volume of water and then run through the Doisy column in the same manner as the original alcoholic mother liquor. The petroleum ether obtained from this extraction contains a large amount of hormone (approximately 25 per cent of the total in some cases) but it is not as pure as that obtained from the original alcoholic mother liquor. In the latter case 1 rabbit unit was equal to 19 to 35 mg. of solids, whereas from this fraction 1 rabbit unit equaled 100 to 125 mg. This is purified in the same manner as the first petroleum ether fractions, but it is not combined with them because the non-crystallizable oils are not as potent. Pure progesterone has been obtained, however, from the fraction.

SUMMARY

An improved procedure for the preparation of progesterone from pig ovaries is described in detail. By this method 25 per cent of the hormone present in the tissue may be recovered in the pure state.

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THE RELATION OF GLYCOGEN, FAT, AND PROTEIN TO WATER STORAGE IN THE LIVER*

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The conflicting views derived from recent attempts (1-7) to relate water and carbohydrate metabolism in the liver leave little doubt that as yet no case has been established for or against the participation of water during the deposition of liver glycogen. The fallacies in the early claim of Zuntz *et al.* (8) that 3 gm. of water accompany the storage of each gm. of glycogen in the liver have been clearly stated by Bridge and Bridges (1) who, though not denying the possibility that some water may accompany glycogen storage, nevertheless point out that factors other than glycogen must operate in determining the water content of the liver. They regard as untenable the view that an exact mathematical relation exists between water and glycogen in the liver (2). A similar view has been expressed by Peters and Lavietes (9) who, from a study of published data, find no support for the "water-binding power of glycogen in the body as a whole." A number of other workers (4-6), however, claim that appreciable amounts of water are associated with the deposition of liver glycogen.

Little definite is known about the rôle of water in the storage of either fat or protein in the liver, although in the case of the former it has been assumed that little or no water accompanies its deposition. The factor commonly employed to estimate water held by "body protein" has been inferred from studies of nitrogen eliminated in the urine during inanition or alterations in nutritional states (10). This experimental approach is indirect and involves numerous doubtful assumptions, the validity of which has been justly questioned by Peters and Lavietes (9).

* Aided by a grant from the Research Board of the University of California.

It was recognized early in this laboratory that the rôle of water in the metabolism of carbohydrate, fat, and protein in the liver could be evaluated only when all four constituents were *simultaneously* determined. This apparently was not realized by all of the earlier workers, some of whom confined their observations to glycogen alone or to glycogen and fat under a relatively limited range of variation (66 to 76 per cent) in the water content of the liver. In the present investigation it was the simultaneous determination of fat, glycogen, and protein under a large variety of conditions in which the lipid content varied from 3 to 66 per cent and the water from 28 to 72 per cent that made possible a clear interpretation of water metabolism in relation to the storage of fat, glycogen, and protein. It was found that the water present in the liver is determined principally by its protein content, both fat and glycogen being deposited in this organ unaccompanied by measurable amounts of water.

EXPERIMENTAL

The data recorded in this study were obtained from 59 adult dogs. In order to obtain as wide fluctuations as possible in the fat as well as in the carbohydrate and protein content of the liver, a large variety of physiological conditions was investigated. The animals studied included (1) normal dogs, (2) depancreatized dogs receiving insulin and various diets, (3) depancreatized dogs deprived of both food and insulin, (4) hypophysectomized dogs, (5) hypophysectomized-depancreatized (Houssay) dogs, (6) a fasted phlorhizinized dog,¹ (7) a thyroid-fed dog.²

The animals received twice daily, at 8.00 a.m. and 4.00 p.m., a diet mixture of lean meat, sucrose, bone ash, and vitamin supplements. The hypophysectomized-depancreatized dogs had access to a fish-cereal mixture and milk in addition to the regular meat diet.

Sampling of Liver—Livers were removed between 8.00 and 10.00

¹ For a period of 6 days prior to removal of the liver, this animal received subcutaneously three times daily 1 gm. of phlorhizin dissolved in 25 cc. of 2.5 per cent sodium bicarbonate.

² For 23 days prior to removal of the liver, this animal received daily 0.5 gm. of desiccated thyroid gland per kilo of body weight in addition to the regular diet.

a.m. under anesthesia induced by the intraperitoneal injection of 50 mg. of amytal (in the form of its sodium salt) per kilo of body weight. The animals, except those fasted, had received their last meal (and last administration of insulin in the case of the depancreatized dogs) at 4.00 p.m. of the previous day. The surface of the liver was wiped clean of blood, and samples were obtained for glycogen determination as described previously (11). Portions were rapidly removed from the different lobes and immersed in 30 per cent potassium hydroxide. The gallbladder was now carefully removed, and the liver weighed and ground in a meat chopper. The grinding was repeated three times to insure thorough mixing. Samples were then taken for the determination of lipids, water, and nitrogen, and placed in glass-stoppered bottles containing 95 per cent alcohol.

Glycogen Determination—Glycogen was determined by the Pflüger method as modified by Good, Kramer, and Somogyi (11). The further modification of Cori and Cori (13), in which the glycogen was dissolved in water and reprecipitated, was also adopted. The reducing power of the glycogen hydrolysate was determined before and after yeast fermentation. The yeast was prepared and washed according to Somogyi's method (14). The improved copper reagent of Shaffer and Somogyi (15) was used for sugar determination. Glycogen is expressed as gm. of fermentable sugar per 100 gm. of tissue.

Total Lipid Determination—The extraction and determination of lipids² have been previously described (16). Hashed liver tissue was extracted with two different portions of alcohol, and the residue was then extracted overnight with ethyl ether in a Soxhlet apparatus. This defatted tissue was carefully dried in a vacuum desiccator and then weighed. The combined alcohol and ether extracts were concentrated *in vacuo*, and the lipids were completely extracted with petroleum ether and made up to volume. Total lipid was determined by evaporating the solvent from an aliquot portion of the petroleum ether extract and weighing the residue. The method employed for determination of total fatty acids has been described elsewhere (16).

Nitrogen Determination—The defatted dried tissue mentioned above was ground to a fine powder in a corn mill, and the total

² The terms lipid and fat are used interchangeably in this communication.

nitrogen of the powder was determined by the macro-Kjeldahl method. Total nitrogen, expressed as percentage of fresh liver tissue, was obtained by multiplying the percentage of nitrogen in the powder by the percentage of defatted dried tissue in the fresh liver. The solvents employed in the preparation of the defatted tissue remove the major portion of the non-protein nitrogen (urea, lecithin, cephalin, etc.), and after drying a powder is obtained, the nitrogen of which is derived almost entirely from protein.

Water Determination—The percentage of water was calculated as 100 minus the sum of the percentages of total lipid and defatted dried tissue. This indirect method avoids loss by volatilization of substances other than water as well as any gain in weight by oxidation of fatty acids, both of which may occur in the direct

TABLE I

Comparison of Direct and Indirect Methods for Water Determination in Liver

The results are expressed in terms of per cent of fresh tissue.

Dog No.	Total lipid	Water	
		Direct method	Indirect method
15	22.9	57.9	57.1
16	31.0	52.6	52.2
28	7.1	67.1	66.6
29	7.0	66.1	65.8

method. It is realized, however, that in the indirect method small amounts of substances such as salts, glucose, etc., may be extracted with alcohol and yet not appear in the petroleum ether extract. In Table I the water contents obtained with the same sample of liver by direct and indirect methods are compared. In the direct method the liver tissue was dried to constant weight in a vacuum oven kept at 80°. The results show that close agreement is obtained between both methods.

Relation of Water to Fat

In Fig. 1 lipids have been plotted against water, both being expressed as per cent of the fresh liver tissue. 59 values are recorded in which lipid values range from 3 to 66 per cent and water from 28 to 72 per cent. The values fall along a straight line of

negative slope. Thus, as the percentage of lipids in the liver increases, that of water decreases. A similar relation with essentially the same slope is obtained when water is plotted against total fatty acids or neutral fat (triglycerides). In order to simplify the treatment of the data, however, only total lipids will be considered throughout.

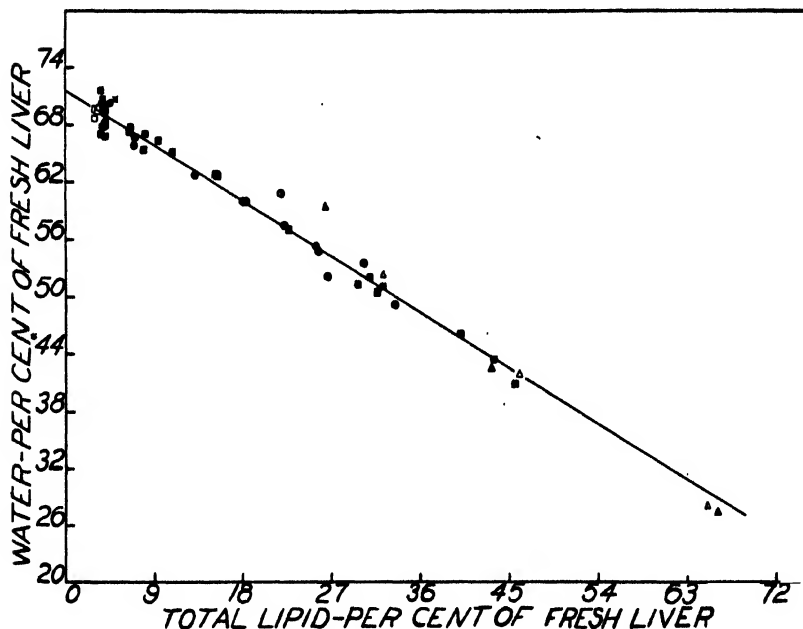


FIG. 1. The relation of water storage to fat deposition in fresh liver tissue. \square represents normal dogs; \blacksquare and \bullet , depancreatized dogs in the post-absorptive state; \triangle fasted depancreatized; \blacksquare hypophysectomized; \blacktriangle hypophysectomized-depancreatized; \odot fasted and phlorhizinized; \otimes thyroid-fed.

In seeking an explanation of the deviations of some of the points from the straight line shown in Fig. 1, attention was directed to glycogen, a constituent that fluctuated from 0.05 to 10.5 per cent in the series of livers recorded. The influence of this variable factor was removed in Fig. 2, in which the values for both water and total lipids have been recalculated on the basis of glycogen-free tissue. The new values were obtained by multi-

Water Storage in Liver

plying the values for fresh tissue by the factor $100/(100 - a)$, where a represents the percentage of glycogen in fresh tissue. A comparison of Figs. 1 and 2 shows that, when the effect of glycogen upon the weight of the liver is removed, the deviation of the ex-

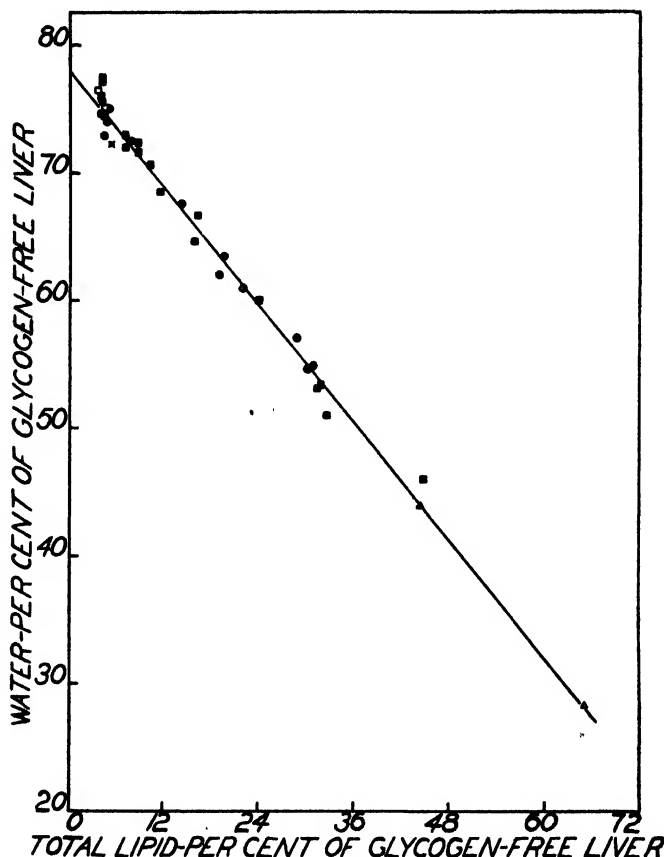


FIG. 2. The relation between liver water and fat in glycogen-free tissue. The symbols have the same meaning as for Fig. 1.

perimental data from a linear relationship is considerably reduced. Indeed, the close approximation of the recalculated values to linearity is striking. Three interpretations may be offered to account for this linear relation between water and lipids.

1. The total water content of the liver remains constant during

the infiltration of fat, a process that increases the size and weight of this organ. In this case a constant amount of water becomes distributed over a greater mass of tissue, and hence the amount of water per unit weight of liver tissue decreases in proportion to the amount of fat that enters the liver. According to this view, fat plays no active part in the water metabolism of the liver, its deposition merely decreasing the percentage of water by increasing the liver weight.

2. A straight line with negative slope might also be observed if a small but definite amount of water were to accompany the storage of lipids in the liver. If this were the case, the increase in lipids would predominate over the increase in water, the net result being that the concentration of water as per cent of the fresh tissue would decrease as fat enters the liver, even though the absolute amount of water increased.

3. As lipids enter the liver, water is displaced, the latter then leaving the liver. If a displacement of water by fat had occurred, however, the slopes of the curves shown in Figs. 1 and 2 would have been much steeper.

It will be shown in the following section that the first hypothesis presented adequately explains the observation that the percentage of water decreases linearly as the percentage of fat increases.

Relation of Water to Glycogen

In Fig. 3 the water and glycogen concentrations in the liver have been reckoned on the basis of fresh tissue. It can be seen that no linear relation exists between the percentages of these substances contained in the liver. This is strikingly brought out in the case of two dogs whose livers were practically devoid of glycogen. The livers of Dogs 37 and 30 contained 0.05 and 0.20 per cent glycogen, whereas their water contents were 60.8 and 28.3 per cent respectively. Thus, even though less than 1 per cent glycogen was present in both livers, one contained twice the water content of the other. A similar independence in the storage of water and glycogen is shown in livers containing *high* percentages of glycogen. Thus the livers of Dogs 19 and 34 contained identical amounts of glycogen, namely 8.4 per cent, but their water contents were 52.2 and 71.7 per cent respectively.

It has been shown in the preceding section, however, that the

percentage of water decreases linearly as the percentage of lipids increases. It is therefore very likely that the accumulation of large amounts of lipids obscures a possible relation between water and glycogen when the percentages of these substances are ex-

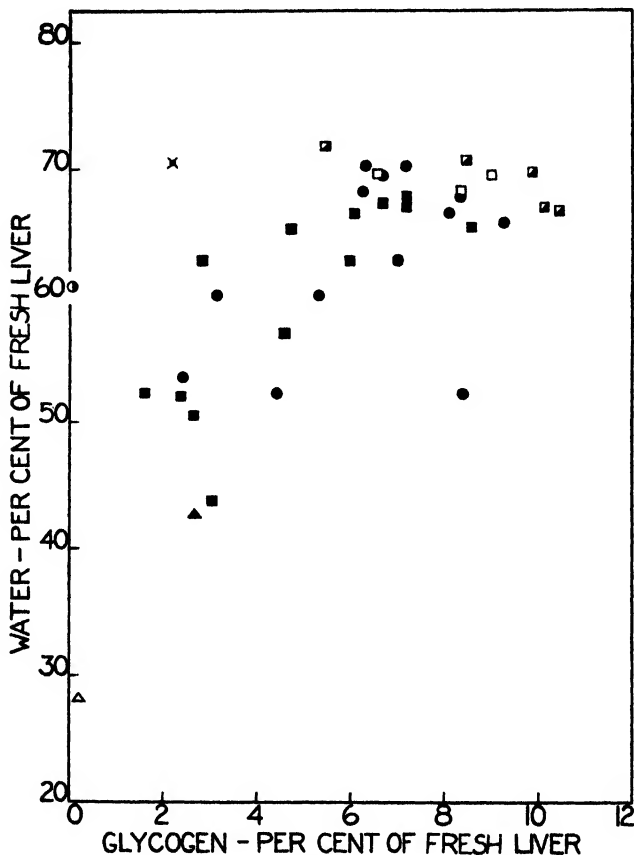


FIG. 3. The relation of water to glycogen in fresh liver. The symbols have the same meaning as for Fig. 1.

pressed on the basis of fresh tissue. But the effect of varying amounts of lipids upon such a relation can be eliminated by referring the values for both water and glycogen to lipid-free liver tissue. This has been done below.

Relation of Water to Glycogen When Both Are Expressed As

Percentages of Lipid-Free Tissue—The values of water and glycogen as percentages of lipid-free tissue were obtained by multiplying their respective concentrations in fresh tissue by the factor $100/(100 - b)$, where b represents the concentration of lipids as percentage of the fresh tissue.

The new values for water are plotted against those for glycogen in Fig. 4, and a linear relation between these two substances is

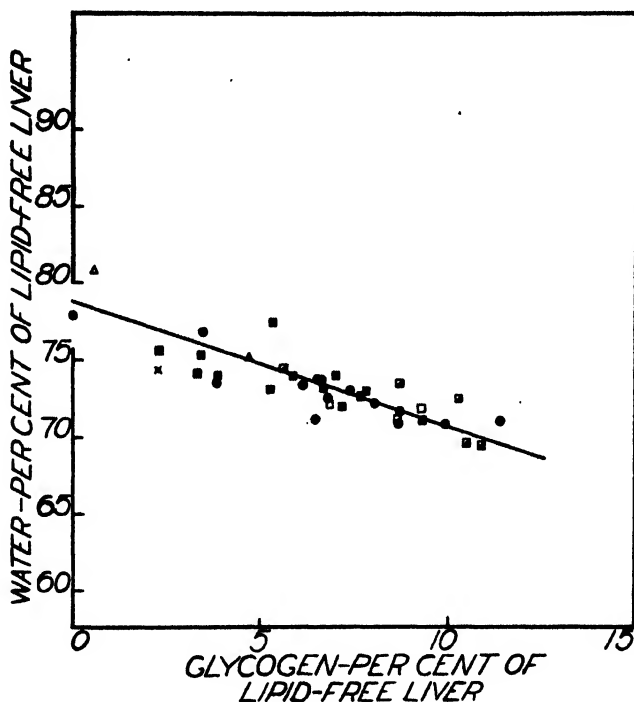


FIG. 4. The relation of water to glycogen in lipid-free tissue. The symbols have the same meaning as for Fig. 1.

obtained; the slope of the curve expressing this relation is negative. Thus, when the masking effect of fat is removed, it is seen that the water concentration in the liver decreases as glycogen increases. Fig. 4 therefore provides strong evidence against the view that measurable amounts of water participate in the storage of glycogen in the liver.

The negative slope of the curve in Fig. 4 does not necessarily

TABLE II
Relation of Water to Protein

Dog No.	Condition	Weight of dog kg.	Weight of liver gm.	As per cent of fresh liver		As per cent of glycogen- free, lipid- free liver		Water Protein	Water R*
				Water	Nitrogen	Water	Nitrogen		
1	Normal	9.1	300	68.3	2.68	78.0	3.06	4.1	3.6
2		7.8	260	69.4	2.78	77.6	3.11	4.0	3.5
3		6.2	270	69.6	2.41	79.4	2.74	4.6	3.8
4	Depancreatized†	6.0	280	67.1	2.36	79.2	2.78	4.6	3.8
5		6.0	400	67.8	2.37	78.6	2.75	4.6	3.7
6		6.6	355	65.2	2.61	77.4	3.09	4.0	3.4
7		7.4	395	52.1	2.04	78.0	3.06	4.1	3.6
8		8.8	485	50.6	2.08	77.0	3.16	3.9	3.4
9		4.6	235	62.8	2.61	76.7	3.19	3.9	3.3
10		7.8	390	62.7	2.16	79.6	2.74	4.7	3.9
11		7.3	490	65.4	2.30	78.4	2.76	4.5	3.6
12		9.8	375	67.2	2.57	77.5	2.96	4.2	3.4
13		5.5	335	66.3	2.28	78.6	2.70	4.7	3.7
14		6.9	750	43.7	1.29	81.6	2.41	5.4	4.5
15		7.0	385	57.1	2.04	78.7	2.81	4.5	3.7
16		8.5	360	52.2	2.09	77.5	3.10	4.0	3.4
17		8.7	640	70.3	2.62	78.9	2.94	4.3	3.7
18		8.5	420	62.7	2.41	78.6	3.02	4.2	3.7
19		10.8	850	52.2	1.86	80.0	2.85	4.5	4.0
20		7.4	480	60.0	2.58	76.6	3.28	3.7	3.3
21		9.1	800	53.6	1.80	79.6	2.68	4.8	3.9
22		7.2	320	67.9	2.74	77.5	3.13	4.0	3.5
23		10.5	400	68.3	2.85	76.1	3.18	3.8	3.2
24		7.8	500	60.0	2.30	78.8	3.02	4.2	3.7
25		9.6	425	52.3	2.04	78.3	3.06	4.1	3.6
26		10.5	320	69.7	2.74	77.8	3.06	4.1	3.5
27		6.9	425	70.2	2.46	78.9	2.76	4.6	3.7
28		8.7	490	66.6	2.35	78.5	2.77	4.5	3.7
29		7.2	365	65.8	2.19	78.6	2.62	4.8	3.7
30	Fasted depancre- atized‡	9.2	700	28.3	0.90	81.3	2.58	5.0	4.3
31	Hypophysectomized	14.0	275	67.1	2.50	77.8	2.90	4.3	3.5
32		13.7	290	71.7	2.68	79.0	2.95	4.3	3.7

* R represents the sum of protein and undetermined substances.

† Last food and insulin received 16 to 18 hours prior to removal of liver.

‡ Neither food nor insulin received for 12 days prior to removal of liver.

TABLE II—*Concluded*

Dog No.	Condition	Weight of dog		Weight of liver		As per cent of fresh liver		As per cent of glycogen-free, lipid-free liver		Water Protein	Water $\frac{R}{R_0}$
		kg.	gm.			Water	Nitrogen	Water	Nitrogen		
33		8.2	210	69.8	2.38	80.9	2.76	4.7	4.2		
34		15.8	350	70.7	2.40	80.5	2.73	4.7	4.1		
35		9.0	235	66.8	2.30	77.9	2.68	4.7	3.6		
36	Hypophysectomized-depancreatized§	5.8	280	42.7	1.60	79.0	2.96	4.3	3.7		
37	Phlorhizinized and fasted	6.2	225	60.8	2.41	78.0	3.09	4.0	3.5		
38	Thyroid-fed	7.5	200	70.6	3.22	76.1	3.47	3.5	3.2		
Mean value.....								78.5	2.92	4.3	3.7
“ deviation.....								1.0	0.19	0.3	0.2

§ Received no insulin for 15 weeks, during which time this animal lost 46 per cent of its initial body weight.

|| Deprived of food for 7 days.

mean that water leaves the liver when glycogen is stored. If the total amount of water in the liver were to remain constant, then, as the liver increased in weight with additional glycogen, the amount of water per unit weight of fresh tissue would decrease. This explanation can be tested by determining the values for water and glycogen in tissue that is both lipid-free and glycogen-free.

Relation of Glycogen to Water When Both Are Referred to Lipid-Free, Glycogen-Free Liver Tissue—The values of water or glycogen as percentages of lipid-free, glycogen-free tissue may be obtained by multiplying their concentrations in fresh tissue by the factor $100/(100 - (a + b))$, where a represents the concentration of glycogen and b the concentration of lipids, both being expressed as percentages of the fresh tissue. The new values for water derived from this calculation appear in Table II.

The relation between water and glycogen when the fluctuating effects of both glycogen and fat upon the weight of the liver are removed is shown in Fig. 5. Despite marked variations in the

glycogen content of the livers (from 0.06 to 12.9 per cent), the concentration of water remains relatively constant. The mean value for the water content of the lipid-free, glycogen-free liver tissue is 78.5 per cent, with maximum and minimum values of 81.6 and 76.1 per cent respectively (Table II). The mean deviation is 1.0 per cent. The very fact that the concentration of water, which varied from 28 to 72 per cent in fresh tissue, can be converted to figures lying within such a narrow range when related to the lipid-free, glycogen-free tissue leaves no doubt that

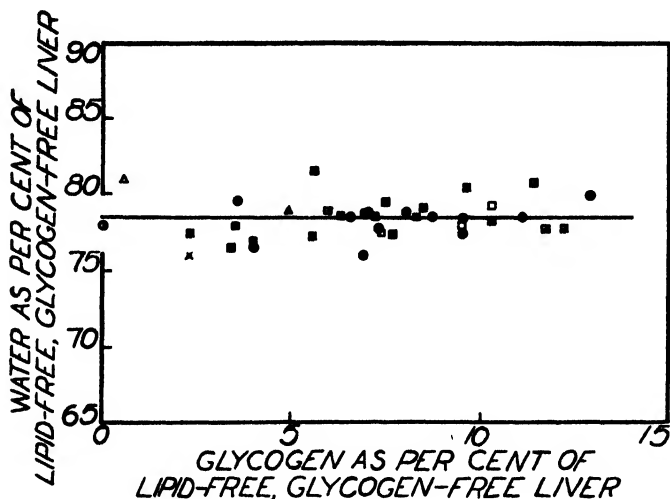


FIG. 5. The relation of water to glycogen in lipid-free, glycogen-free tissue. The straight line has been drawn at the arithmetical mean for water, namely 78.5 per cent. The symbols have the same meaning as for Fig. 1.

liver water is independent of both glycogen and fat. This also makes it clear that the negative slopes observed in Figs. 1, 2, and 4 are entirely due to the influence of increasing amounts of lipids or glycogen, as the case may be, upon the weight of the liver, for as these substances enter the liver a relatively constant amount of water becomes distributed over a larger mass of tissue, and this results in a decrease in the percentage of water.

Significance of the Constant 0.785—In each of thirty-eight livers water, glycogen, lipids, and protein were separately determined.

Residual materials were obtained by difference.⁴ The influence of glycogen and lipids upon the weight of the liver was then removed, and the ratio of water to the remaining mass (lipid-free, glycogen-free tissue) was noted. This ratio, *viz.* water/(water + protein + undetermined substances), was found to be sensibly constant in every case, the average value being 0.785. The constancy of this ratio of course implies the constancy of the ratio, water/(proteins + undetermined substances). Thus, if we represent the percentage of the water by W and the percentage of proteins plus undetermined substances by R , the first equation becomes

$$\frac{W}{W + R} = k \quad (1)$$

whence

$$\frac{W}{R} = \frac{k}{1 - k} \quad (2)$$

* Evidently the constancy of either $W/(W + R)$ or W/R implies that the deposition of glycogen and fat in the liver does not increase the water content. For, suppose that the addition of g gm. of glycogen and fat brings in z gm. of water, then the subsequent mass of water would be $W + z$. The ratio $W/(W + R)$ now becomes $(W + z)/(W + z + R)$, which cannot be equal to the original value, namely $W/(W + R)$, unless $R = 0$, which is obviously never the case.

Table II shows the value of the ratio $100 (W/(W + R))$ in the case of each of the thirty-eight liver samples examined. It should be noted that the maximum value was 81.6 and the minimum 76.1. The mean value was 78.5 with a mean deviation of 1.0.

If each liver sample is taken on the basis of 100 gm. of fresh weight, with W denoting the percentage of water and S the percentage of combined glycogen and fat, the equation

$$\frac{W}{100 - S} = 0.785, \text{ or } W = 0.785 (100 - S) \quad (3)$$

⁴ Undetermined substances (salts, etc., including additive errors) were calculated as $100 - (\text{water} + \text{glycogen} + \text{total lipids} + 6.24 \times \text{nitrogen})$. The values for undetermined substances were found to range from 0.9 to 4.4 per cent, with a mean value of 2.6 per cent.

is obtained. This linear relation is shown in Fig. 6. The agreement of the various samples with the average value of 0.785 for the ratio is striking.

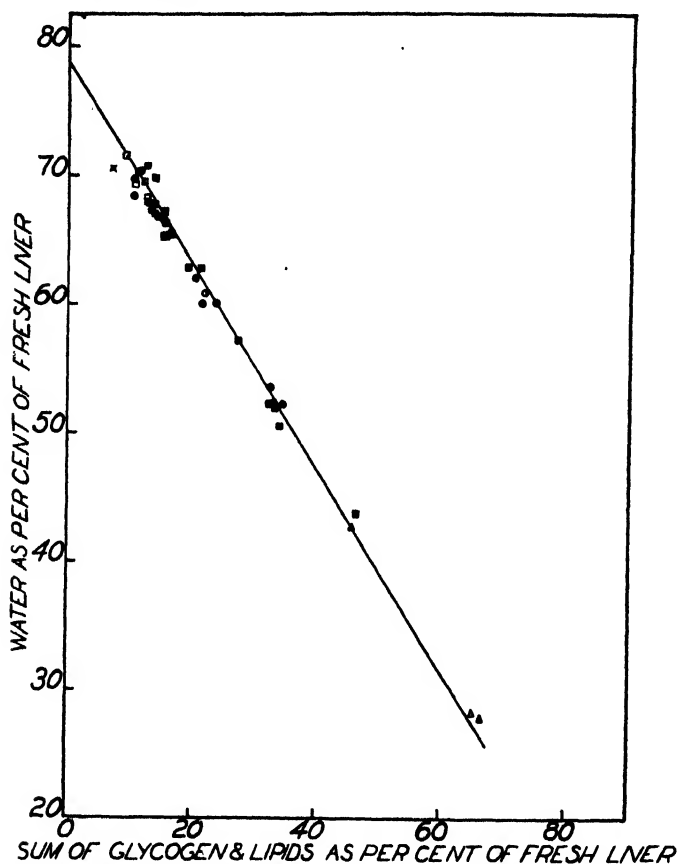


FIG. 6. The relation of water to the sum of glycogen and fat in the liver. The straight line is the graph of Equation 3, viz. $W = 0.785 (100 - S)$, where W represents the percentage of water and S the sum of the percentages of glycogen and fat. The symbols on the curve have the same meaning as for

A similar linear relation between W and R is

$$W = \frac{0.785}{1 - 0.785} R \quad (4)$$

In other words, the water content of the liver varies directly as R ; i.e., as the substances other than fat and glycogen. Proteins and salts constitute by far the major portion of R , and these will be dealt with in the following section.

Relation of Water to Protein Content of Liver

Thus far it has been definitely established that a factor (or factors) other than fat or glycogen determines the storage of water in the liver. It has also been shown that the water content of the liver is a function of an analytical fraction that included protein and certain undetermined substances such as salts, etc. The relation of water to one of these, i.e. protein, will now be considered.

If a basic relation between water and protein exists, this should appear by removing the masking effects of both glycogen and fat. In Table II, therefore, the concentrations of both water and nitrogen for each liver have been recalculated and refer to a lipid-free, glycogen-free liver. As already pointed out in another connection, Table II shows that, when the masking effects of both fat and glycogen have been removed, liver tissue has a relatively constant composition in respect to water. In the lipid-free, glycogen-free tissue, the percentages of nitrogen ranged from 2.41 to 3.47. The average value was 2.92 per cent, and the mean deviation from this value was 0.19 per cent. If the two highest and two lowest values are excluded, however, the nitrogen values ranged from 3.2 to 2.7 per cent. The occurrence of the relative constancies in both water and nitrogen in the presence of huge fluctuations in both lipid and glycogen suggests that protein and water are interrelated. This is borne out by Fig. 7, where the total amounts of water and nitrogen in the entire livers of forty-seven dogs have been plotted. In view of the wide fluctuations found in the size of the livers as well as in their nitrogen contents, Fig. 7 provides suggestive evidence that the water content of the hepatic tissue bears a direct linear relation to its protein content. The fact that, with some exceptions, the values shown in Fig. 7 fall along a straight line becomes all the more significant when it is realized that the forty-seven livers recorded in this figure were obtained under such diversified experimental conditions as those found in depancreatized, phlorhizinized, hypophysectomized, thyroid-fed

as well as normal dogs, all of which provided variations in liver weights from 200 to 850 gm., in fat content from 7.9 to 326 gm., in glycogen content from 0.1 to 71.5 gm., and in nitrogen content from 4.5 to 16.8 gm.

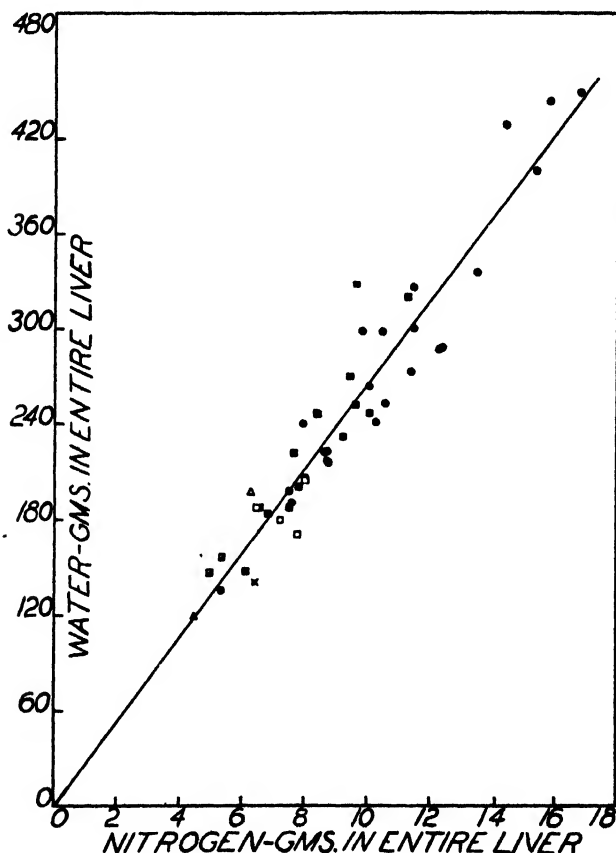


FIG. 7. The relation between absolute amounts of water and nitrogen in the entire liver. The symbols have the same meaning as for Fig. 1.

The amount of water associated with each gm. of liver protein in the dog may now be *roughly* approximated. In this investigation, total nitrogen was determined on liver tissue that had previously been extracted with alcohol and ether. By virtue of their solubility in these solvents, the major portion of the non-

protein nitrogen constituents, such as urea, lecithin, and cephalin, was removed from the fraction employed for determinations of total nitrogen. Thus the nitrogen values shown in Table II represent the nitrogen derived almost entirely from protein. In Table II the ratios of water to protein have been calculated for the livers of thirty-eight dogs. The factor obtained recently by Addis *et al.* (17) has been used for converting nitrogen to liver protein. The average value for the water to protein ratio was found to be 4.3, with a mean deviation of 0.3.

Less variation was observed in the ratios of water to protein plus undetermined substances than in the ratios of water to protein alone (Table II). A significant fraction of the undetermined substances is salt, a constituent that obviously plays a part in water metabolism. The closer agreement among the values of the ratios for water to protein plus undetermined substances undoubtedly indicates that the combined influence of proteins and salts rather than of protein alone is the factor that determines the water content of the liver. With few exceptions, the agreement between individual values and the average value of the ratio, $\text{water}/(\text{protein} + \text{undetermined substances})$, is not unreasonable. The deviations from the average, however, may, in part, be accounted for by the fluctuations in the amounts of blood and bile contained in the liver after its removal from the body, since the ratio of water to solids in these two fluids differs from that of the hepatic cell proper. Although these deviations invite further study, it is clear from the present investigation that protein is the most significant single factor determining the water content of the liver.

The results obtained in this study do not warrant the conclusion that glycogen and lipids are deposited in an anhydrous state. If traces of water accompanied the storage of these substances, the net change in water would be too small for experimental determination. This is particularly true in the case of glycogen, where percentage changes are usually not above 10 per cent. Thus, if 0.1 gm. of water accompanied the storage of each gm. of glycogen, a change from 1 to 11 per cent of glycogen in the liver would result in an increase of about 1 per cent in the water concentration, an amount which is obviously close to the limits of experimental error. In regard to fat, however, our results make it clear that

the commonly accepted figure, namely 0.1 gm. of water per gm. of fat stored, is far too great. The livers of dogs that contain large amounts of lipids are suitable for testing this figure, since

TABLE III*
On the Water Factor in Storage of Lipids

Dog No.	Weight of dog	Total lipid		Glycogen		Observed water	Calculated water			
		Per cent of fresh liver	Total amount in liver†	Per cent of fresh liver	Total amount in liver†		0.0 gm. water per gm. lipid‡		0.1 gm. water per gm. lipid§	
							Per cent of fresh tissue	Difference from observed water	Per cent of fresh tissue	Difference from observed water
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	
	<i>kg.</i>		<i>gm.</i>		<i>gm.</i>			<i>per cent</i>		<i>per cent</i>
7	7.4	30.9	122	2.39	9.4	52.1	52.4	+0.6	55.5	+6.5
8	8.8	31.6	153	2.67	12.9	50.6	51.6	+2.0	54.8	+8.3
14	6.9	43.5	326	3.04	22.8	43.7	42.0	-3.9	46.4	+6.2
16	8.5	31.0	112	1.60	5.8	52.2	52.9	+1.3	56.0	+7.3
19	10.8	26.4	224	8.41	71.5	52.2	51.2	-1.9	53.8	+3.1
21	9.1	30.3	242	2.44	19.5	53.6	52.8	-1.5	55.8	+4.1
25	9.6	28.8	122	4.41	18.7	52.3	52.4	+0.2	55.3	+5.7
30	9.2	65.0	455	0.20	1.4	28.3	27.3	-3.5	33.8	+19.4
36	5.8	43.2	121	2.35	6.6	42.7	42.7	0.0	47.0	+10.1

* Only those livers containing more than 25 per cent lipids are included in this table.

† The amounts of total lipid and glycogen found in livers of normal dogs weighing from 6.2 to 14.3 kilos were, respectively, 7 to 12 and 10 to 25 gm.

‡ The water values in Column 7 are calculated from Equation 3, namely $W = 0.785 (100 - S)$, where W represents the per cent of water and S the sum of the per cents of glycogen and lipids. These values have been calculated on the basis that no water accompanies the storage of either glycogen or lipids.

§ The water values in Column 9 are calculated on the basis that 0.1 gm. of water accompanies the deposition of each gm. of lipids and that no water accompanies glycogen storage. These values are obtained from the equation $W = 0.785 (100 - S) + 0.1b$, where W and S represent the same constituents as above and b represents the per cent of lipids which are shown in Column 2. In other words, Column 9 = Column 7 + $0.1 \times$ Column 2.

the amount of water accompanying the deposition of considerable amounts of lipids would now be in excess of allowable error. In Table III the possibility that 0.1 gm. of water accompanies each

gm. of fat has been tested. In view of the disagreement between the observed and the calculated values, it would seem that even this small amount of water does not attend the storage of each gm. of fat.

Effect of Fatty Infiltration upon the Glycogenic Function of Liver

Since Rosenfeld's early work (18) it has been assumed that an antagonism exists between the amounts of fat and glycogen present in the liver. While it is undoubtedly true that in a number of abnormal nutritional states as well as in diabetes an increase in fat may occur simultaneously with the depletion of liver glycogen, it seems unnecessary to invoke a metabolic antagonism between fat and glycogen to account for the amounts of these substances present in the liver in conditions such as those produced by poisoning with arsenic, phosphorus, and chloroform. The depletion of glycogen in toxic conditions is doubtless the result of injurious effects on the liver cell, which interfere with its glycogenic function and at the same time cause an alteration in the cell characterized by fatty degeneration.

The lipid and glycogen content of livers found in the present investigation to contain more than 25 per cent fat is recorded in Table III. Since the liver enlarges as fat accumulates, it is apparent that the true capacity of the liver to store glycogen can be gaged only by considering the total amount of glycogen present in the entire organ. This has been done in Table III. Although it was found that a high lipid content in the liver may be associated with a low glycogen content (*e.g.* phlorhizin), the results of this investigation show quite clearly that the infiltration of large amounts of fat *need* not interfere with the glycogenic function of the liver. This is particularly well brought out in the case of two dogs, Nos. 14 and 19. Normal amounts of glycogen, and even higher, were present in these livers, in which a 46- and 32-fold increase in fat had occurred. It may be concluded, therefore, that so long as the liver cell is not injured by toxic substances the presence of large amounts of fat does not interfere with the capacity of the liver cell to store normal amounts of glycogen.

The assistance of Mr. G. E. Gibbs, Mr. C. Entenman, and Mr. G. Changus in carrying out some of the analyses is gratefully acknowledged.

SUMMARY

1. A study was made of the water content of dog liver in relation to glycogen, fat, and protein in a large variety of experimental conditions, which included' depancreatized, hypophysectomized, hypophysectomized-depancreatized, phlorhizinized, thyroid-fed as well as normal dogs. These conditions provided fluctuations in lipids from 3 to 66 per cent, in glycogen from 0.05 to 10.5 per cent, in water from 28 to 72 per cent, and in nitrogen from 0.90 to 3.22 per cent.

2. Measurable amounts of water do not accompany the storage of glycogen in the liver.

3. Measurable amounts of water do not accompany the deposition of fat in hepatic tissue.

4. Although neither glycogen nor lipids affect the *absolute* water content of the liver, the *percentage* of water in this tissue decreases linearly as the sum of the percentages of lipids and glycogen increases.

5. The water content of the liver is directly proportional to an analytical fraction, the chief constituent of which is protein.

6. The presence of large amounts of fat need not interfere with the capacity of the liver cell to store normal amounts of glycogen. This observation does not support the view of a metabolic antagonism between glycogen and fat in hepatic tissue.

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A COMPARISON OF THE ANTITRYPTIC ACTIVITY OF EGG WHITE WITH ITS CAPACITY TO PRODUCE A CHARACTERISTIC NUTRITIONAL DISORDER*

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The suggestion was early made¹ that the inhibitory action of egg white toward digestion might account for various physiological effects of raw egg white. More recently, Cohn and White (4) have postulated antitrypsin as one of the possible factors in the etiology of the specific pellagra-like syndrome produced in various animals by including in the ration egg white that has not received thorough heat treatment.

It had been considered improbable, by the present author, that a protein lack due to antienzyme activity was involved in this nutritional disorder, not only because large amounts of casein or thoroughly cooked egg white added to the basal ration containing raw egg white had failed to cause any improvement in the symptoms in this laboratory, but also because the amount of heat treatment necessary to render certain lots of fermented egg white harmless (6) seemed incompatible with the known labile nature of enzymes. However, the report both from the Yale laboratory and that of Balls (2) that antitrypsin is stable to relatively drastic heat treatment made the latter argument less pertinent. Balls and Swenson (1, 2) extracted a tryptic inhibitor from thin egg white with dilute ammonia and concentrated it as much as 150

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¹ The literature has been reviewed by Bateman (3).

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times. They believe it to be not a true protein but a hydrolysis product of protein and to act by combining reversibly with inactive trypsin or else with kinase. Thus the utilization of cooked egg white or casein added to the basal ration could conceivably be interfered with by the tryptic inhibitor present in the raw egg white. Inasmuch as the capacity of egg white to induce the nutritional disorder was found, in this laboratory, to persist after a 30 day treatment with 70 per cent ethyl alcohol, an inquiry was sent to Dr. Balls of the Food Research Division at Washington concerning the effect of this process on his concentrate, with a resultant exchange of preparations between the two laboratories for testing.

EXPERIMENTAL

Commercial, fermented dried egg white was hardened with ethyl alcohol as noted in Table I, leached in water for varying lengths of time, dried, ground in a ball mill, and an extraction of the inhibitor was made according to the method of Balls and Swenson (2) with the following modifications: The heating of the dilute ammonia extract was omitted inasmuch as nearly all of the coagulable protein was already removed; and no further purification was undertaken after the precipitation of the crude inhibitor with alcohol.

The antitryptic activity was determined by the method² of Willstätter and coworkers (7) with the following modifications: Merck's pancreatin was used as the source of trypsin and a sample of casein purified in this laboratory in place of Hammarsten's. Digestion of the samples was carried on for an hour and a half instead of for 20 minutes, with the idea that this lengthening of the interval might tend to equalize more fairly the influence on digestion of the inhibitor added as a concentrate and that added as a mixture with the residues or egg white. For the latter as well as the former, control blank experiments were performed by titrating similar samples of each without digestion. The disappearance of the inhibitor in Lot I on autoclaving Lot G gives a satisfactory check on the adequacy of these blanks.

² The author is indebted to Dr. Marvin J. Johnson of the Department of Agricultural Bacteriology and Agricultural Chemistry, University of Wisconsin, for suggestions on certain of the procedures.

TABLE I
Antitryptic Content and Physiological Activity of Some Egg White Preparations

Lot	Material tested	Weight of sample	Degree of inhibition of trypsin of sample	Result of test of physiological effect of material on rats
		mg.	per cent	
A	Concentrated inhibitor from fresh, thin egg white from Dr. Balls' laboratory	2.5	37	Fed as 5% of ration. In 2 wks. soreness of mouth healed; new hair on lips, abdomen, and paws
		5.0	44	
		10.0	46	
		20.0	53	
		40.0	60	
B	Inhibitor prepared by modified Balls-Swenson method from fermented, dried egg white after 3 days in 95% EtOH and leaching in water	2.5	16	Fed as 10% of ration. In 2 wks. severe condition at beginning healed; new hair on back and lips
		5.0	20	
		10.0	29	
		20.0	42	
		40.0	48	
C	Inhibitor prepared as for Lot B	5.0	25	Fed as 10% of ration. Results as for Lots A and B in 2 wks.; continued to end of 4th wk.; became entirely normal
		10.0	34	
		20.0	44	
		40.0	58	
D	" "	20.0	38	Fed as 10% of ration. Practically normal in 3 wks.
		40.0	48	
E	Raw, crude, thin egg white; dried	31.0*	50*	Fed as 40% of diet. Both thin and thick fractions of egg white produced equally severe skin and nerve symptoms
F	Raw, fermented, dried egg white	500	70	At 20, 30, 40, or 66% of basal ration this product intensifies dermatitis, soreness of lips and feet, and allows development of severe nerve symptoms in standard test animals. Fed as 5%, standard symptoms somewhat improved, not as much as on 5% of Lot A

* These figures were taken from the paper by Balls and Swenson (2) for comparison, as the methods used were essentially the same in the two laboratories. The thin and thick egg white samples, the physiological effects of which are recorded, were fed 3 years previously and hence were not available at this time for tests on antienzyme content.

TABLE I—*Concluded*

Lot	Material tested	Weight of sample	Degree of inhibition of trypsin of sample	Result of test of physiological effect of material on rats
		gm.	per cent	
G	Undissolved residue after preparation of Lot B	500	41	Fed as 40% of ration. Rat died after 20 days with very severe dermatitis, soreness of lips and skin, and severe nerve symptoms
H	Lot G after 3 further extractions with dilute ammonia	500	21	Not fed
I	Lot G after autoclaving 15 min. at 15 lbs. pressure	500	4	" "
J	Fermented, dried egg white after treatment with 70% EtOH for 30 days and thorough leaching in water	500	27	Fed as 40%. Rat died after 25 days on ration with very severe dermatitis, soreness of lips and skin, and nerve symptoms
K	Inhibitor prepared from Lot J by Dr. Swenson in laboratory of Dr. Balls	10†	27.5†	Not fed
		20†	50.0†	

† These figures were made available through the kindness of Dr. Balls and Dr. Swenson of the Food Research Division of the United States Department of Agriculture, Washington.

The standard curative technique previously described for testing the physiological effects of egg white on rats (6) was used. The ration for testing the inhibitors and residues listed in Table I was as follows: egg white preparation 5, 10, or 40 per cent, rice polish 10, purified casein 20, Osborne and Mendel's (5) salt mixture 4, equal parts of sucrose and corn-starch, to 100 per cent. From 3 to 5 drops of cod liver oil per rat per day were administered separately.

DISCUSSION

It will be seen from the results in Table I that there is no correlation between the presence of antitrypsin in egg white

products and their capacity to intensify the characteristic pellagra-like disorder. In the tests made, each of the four preparations of inhibitor gave ample evidence of the absence of any significant injurious effect, in striking contrast to other of the samples of egg white products with a much lower antienzyme content.

For example, the egg white residue, Lot G, contained only about $1/25$ as much antitryptic activity as Lot B, the inhibitor extracted from it, or about $1/160$ as much as the partially purified Lot A of Dr. Balls; hence, although Lot G was fed at a 40 per cent level and Lots B and A at 10 and 5 respectively, the injurious ration, incorporating Lot G, contained only $\frac{1}{2}$ and $1/20$ as much antitrypsin respectively, as the other two rations shown to be harmless. There was even a greater lack of correlation in regard to Lots A and J.

This evidence against the hypothesis that the egg white syndrome is due to a lack of protein for which an enzyme inhibitor is responsible is in harmony with the strikingly curative effects of injecting a potent extract containing only a small amount of solids, a report of which is being published elsewhere.

SUMMARY

Crude concentrates of an antitrypsin have been prepared from a commercially fermented dried egg white which has been shown to be highly active in producing pellagra-like lesions in rats and other animals.

These concentrates showed no corresponding increase in the capacity to produce the nutritional disorder, but on the other hand permitted the healing of the characteristic lesions when the diets incorporating them held a much higher concentration of antitrypsin than did the physiologically injurious diets carrying the egg white or extracted residues.

It is therefore concluded that the pellagra-like syndrome due to egg white is not attributable to the antitryptic content of this substance.

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HYDROXYLATED ACIDS OF FATS: AN IMPROVED METHOD OF DETERMINATION*

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A method of determining the acetyl values of lipids by acetylation and titration has recently been described by West, Hoagland, and Curtis (1). The procedure was found to be applicable to free hydroxylated acids and to possess certain other advantages over previous methods. The writers have introduced simplifications and improvements into the technique of the method which make it much more convenient to use and more accurate. The method has been applied to a study of the acetyl values of a number of fats and their insoluble acids. We believe the data given below represent the first to be published relative to the hydroxylation of the free insoluble fatty acids of a number of fats obtained by an analytical procedure suitable for the purpose. According to Lewkowitsch (2) "... most of the numbers contained in the older literature and stated to indicate the presence of hydroxylated fatty acids in natural oils and fats ... must be rejected as fictitious." In so far as we are aware, the later literature has contributed little to change this opinion. We have found the insoluble fatty acids of a number of vegetable and animal fats to possess small (except castor oil) but definite acetyl values, indicating a rather general occurrence of small amounts of hydroxylated fatty acids in the common fats. Our method of analysis is being applied to a study of the distribution of hydroxylated fatty acids in the lipids of animal tissues in order to ascertain, if possible, the physiological rôle of these substances.

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*Method**Reagents—*

Acetic anhydride, pyridine, and butyl alcohol as previously given (1).

0.5 N alcoholic KOH, aldehyde-free, is prepared according to Malfatti (3).

Acetylating mixture. 1 volume of acetic anhydride and 7 volumes of pyridine.

*Apparatus and Titration—*The reaction tubes were 25 × 200 mm. Pyrex test-tubes; the condenser tubes, 18 × 150 mm. Pyrex test-tubes with a rubber ring under the rim. 1½ inch holes are drilled in a piece of heavy sheet metal or painted reenforced board and this is placed over the steam bath. Rubber rings are fitted near the bottoms of the reaction tubes so they may be placed through the holes of the steam bath cover with the bottoms of the tubes extending about 5 cm. below the cover into the bath. The pipettes were as previously described (1). A 25 ml. burette graduated in 0.05 ml. with a 20 gage stainless hypodermic needle fitted to the tip was employed. This tip delivers about 0.007 ml. of 0.5 N KOH per drop. The writers use the titration apparatus described by West (4) which utilizes a vacuum windshield motor for driving the stirrer in the reaction tubes. A hand stirrer consisting of a small glass rod with a coil or loop at the bottom and bent at right angles at the top may also be used.

Procedure

Samples for analysis are weighed into two dry reaction tubes (0.5 to 1.0 gm. of materials with high acetyl values and 1.0 to 1.5 gm. of those with low values). 5.0 ml. of acetylating mixture are carefully pipetted into one tube and 5.0 ml. of pyridine alone into the other. A third tube is charged accurately with 5.0 ml. of the acetylating mixture only. The condensers, carefully filled with water and dry on the outside, are suspended in the reaction tubes which are then placed in the holes of the board on the steam bath and heated 1½ hours. The condensers are then slightly raised and 5 ml. of water are added to each reaction tube; the condensers are replaced and the tubes heated 15 minutes longer with occasional careful shaking. After cooling, the condensers and the reaction tubes are rinsed down with 15 ml. of butyl alcohol

and the titrations carried out in the reaction tubes as described above with the alcoholic alkali, 4 drops of 1 per cent phenolphthalein being used as indicator. The titrations of duplicate blanks should not vary more than 0.02 ml. The end-points are exceedingly sharp. The calculations are as given by West, Hoagland, and Curtis (1). All values in the present paper are calculated to represent the mg. of acetyl bound per gm. of sample.

Checks on Method

Lewkowitsch (5) demonstrated that the error in the method of Benedikt and Ulzer (6) for the determination of hydroxylation of insoluble fatty acids is due largely to the formation of relatively stable mixed anhydrides formed upon boiling the acids with acetic anhydride. This source of error also undoubtedly enters into the acetyl values of fatty acids and rancid fats determined by more recently reported methods. The experiment reported below seems to show that our method is not subject to this error.

Two series of acetyl determinations were made on oleic acid with the technique of the improved method, but with substitution of dioxane for pyridine as the medium for the reaction. Samples of oleic acid were set up with the acetylating mixture, heated, and the excess reagent decomposed with water in the usual way. In one series, 5 ml. of pyridine were added to the mixture immediately after the decomposition of the reagent with water. In the other series, no pyridine was added. Following this treatment, both series were further heated and samples removed at intervals of 5 minutes for titration. The results are summarized in Table I.

The samples which had not been treated with pyridine gave evidence, by their apparent acetyl values, of the formation of anhydrides which are quite resistant to decomposition by water at elevated temperatures. The samples which had been treated with pyridine, however, almost immediately showed minimum values. Pure oleic acid should not show any acetyl value, since it contains no hydroxyl groups. The small value obtained for the sample in question is no doubt due to oxidation products in the oleic acid, since neutral equivalent determinations on this sample indicated its molecular weight to be 291 instead of a theo-

retical 282. As pointed out later, free fatty acids upon standing for some time often show increased acetyl values (Table IV).

The applicability of the method to free hydroxylated acids was checked by acetyl determinations on ricinistearolic acid prepared according to Mangold (7) and recrystallized four times from a mixture of 1 volume of ethyl ether and 4 volumes of petroleum ether (30–60°). The compound melted at 50°. Neutral equivalent: found, 295, 296, 296; theory, 296. Acetyl value: found, 146, 145.4, 146, 145.6, 146, average 145.8; theory, 145.3.

TABLE I

Effect of Pyridine on Decomposition of Higher Fatty Acid Anhydrides

Time of heating after decomposition of excess reagent	Acetyl value without pyridine	Acetyl value with pyridine
min.		
5	69.5	1.0
10	48.8	0.4
15	44.4	0.5

TABLE II

Comparison of Official Method of Association of Official Agricultural Chemists (8), Roberts-Schuette Method (9), and Improved Method When Applied to Known Mixture of Castor Oil, Oleic Acid, and Butyric Acid

Method	Acetyl value of castor oil	Theoretical acetyl value of mixture	Acetyl value of mixture as determined
Official.....	126.3	72.1	14.6
Roberts-Schuette.....	127.1	72.5	78.8
Improved.....	125.6	71.6	71.9

In a comparative study of methods of determining the acetyl values of fats and oils, analyses were made of various oils by several methods. Discordant results prompted an attempt to find the cause of the discrepancies. Several factors may be concerned. One of these seems to be the presence of more or less free acidity in the samples analyzed. This was shown to be a probability by running analyses on a known mixture of fats containing a high proportion of free fatty acids. Any error introduced by this factor would under such conditions be magnified and easily recognized.

For this purpose, a mixture of castor oil, oleic acid, and butyric acid was prepared containing 57.1, 28.6, and 14.3 per cent by weight of the constituents, respectively. With the acetyl values of castor oil as determined by the different methods and the percentage composition of the mixture known, the theoretical acetyl value of the mixture for a given method was then compared with

TABLE III
Acetyl Values and Acidity Values of Fats and Oils

Fat or oil	Acetyl value				Acidity value	
	1st analysis	Average	2nd analysis	Average	1st analysis	2nd analysis
Butter.....	2.8, 2.9, 3.0	2.9	2.5, 2.9	2.7	0.32	0.32
Castor.....	125.5, 125.5, 125.7	125.6			0.40	
Coconut.....	1.8, 1.9, 1.8	1.8	1.2, 1.4	1.3	0.04	0.07
Cod liver.....	2.0, 2.5, 2.3, 2.4, 2.5	2.3	3.8, 4.5	4.2	0.32	0.50
Corn.....	3.0, 2.9, 3.0	3.0	3.7, 3.5	3.6	0.09	0.15
Cottonseed 1.....	4.0, 3.9, 4.0	4.0	5.1, 5.2	5.2	0.04	0.19
“ 2.....	4.5, 4.2	4.4			0.11	
Lard.....	0.9, 0.9, 1.0	0.9	1.5, 1.5	1.5	0.24	0.33
Linseed raw.....	4.5, 4.5, 4.6	4.5	5.3, 5.3	5.3	0.45	0.54
“ boiled.....	5.3, 5.9, 5.9, 6.1, 6.1, 6.2	5.9	7.6, 8.1	7.9	0.73	0.88
Neat's-foot.....	7.3, 7.1, 7.3	7.2	9.2, 8.7	9.0	2.11	2.24
Olive 1.....	4.1, 4.1, 4.1	4.1	13.4, 13.3	13.4	0.54	1.25
“ 2.....	3.2, 3.0	3.1			0.17	
Peanut.....	2.5, 2.7, 2.5	2.6	2.6, 2.6	2.6	0.14	0.19
Salmon.....	3.7, 3.7, 3.7	3.7			0.66	

The acidity value used is defined as the number of cc. of 0.1 N NaOH required to neutralize the free fatty acids present in 1 gm. of the fat (dissolved in butyl alcohol). The second analysis was made 7 months after the first.

that obtained by actual analysis of the mixture. The results, calculated according to the West definition of acetyl value, are given in Table II.

Good agreement with the theoretical value was obtained only with the improved method. In the Official method (8), the loss of soluble acids in the washing process after acetylation probably

constituted the main source of error. Two factors very likely affected the results by the Roberts-Schuetz method: first, incomplete decomposition of higher fatty acid anhydrides; and second, difficulties in titrating insoluble fatty acids in an aqueous medium.

Acetyl Values of Fats and Fatty Acids

The acetyl values of a number of fats and oils were determined and about 7 months later the determinations were repeated.¹

TABLE IV
Acetyl Values of Fats and Their Insoluble Fatty Acids

Fat	Acetyl value of fat	Acetyl value of fatty acids			
		1st analysis	Average	2nd analysis	Average
Butter.....	2.7	1.3, 1.4, 1.5	1.4	12.2, 11.9	12.1
Castor.....	125.6	135.4, 135.1, 136.1*	135.5		
Coconut.....	1.3	0.3, 0.7, 0.2	0.4		
Corn.....	3.6	2.3, 2.0, 1.6	2.0	13.2, 13.7, 13.2	13.4
Cottonseed.....	4.4	2.6, 3.0, 2.9	2.8	20.8, 20.5, 19.5	20.3
Lard.....	1.5	0.9, 0.5, 0.7	0.7	6.8, 6.4, 6.3	6.5
Linseed raw.....	5.3	3.6, 3.5, 3.1	3.4		
“ boiled.....	7.9	4.3, 3.7, 3.7	3.9		
Neat's-foot.....	9.0	4.5, 3.7, 3.3	3.8	5.3, 5.0, 5.3	5.2
Olive.....	3.1	0.6, 0.5, 0.2	0.4	9.5, 10.2, 10.0	9.9
Peanut.....	2.6	2.1, 2.5, 2.5	2.4	19.8, 19.9, 19.6	19.8
Salmon.....	3.7	2.2, 2.2, 2.2	2.2		

The first analysis was made immediately after the preparation of the acids; the second analysis was made after 2 months.

* Values for the fatty acids of castor oil are calculated on the basis of the saponifiable acidity. This was done to correct for ester formation.

These values together with the acidity of the fats and oils are given in Table III. As expected, an increase in acidity is generally accompanied by an increased acetyl value, owing probably in large part to the glycerol liberated.

In Table IV are given the acetyl values of a number of fats and of their insoluble fatty acids.² It will be noted that in a number

¹ The fats and acids were stored in the dark at room temperature.

² In the preparation of the free fatty acids the soap solutions were extracted by Wilkie's method (10) for the removal of unsaponifiable matter.

of instances the acetyl values of the fats are much higher than those of their insoluble acids. This is probably due to the presence of sterols and partly hydrolyzed glycerides in the fats. By comparing the data of Tables III and IV, it will be seen that the free fatty acids are much more prone to autoxidize and produce hydroxylated acids than are the fats. In some instances, namely the acids of butter fat, corn oil, cottonseed oil, olive oil, and peanut oil, the tendency is especially marked. The values given in Tables III and IV represent all of the analyses run and show the variations of duplicate analyses to be generally less than 0.5 of an acetyl unit. Stated otherwise, 0.5 mg. of acetyl bound per gm. of fat or fatty acid can be detected by the method.

SUMMARY

1. An improved method for the determination of the acetyl values of fats and of their free insoluble acids is described.
2. It has been shown, apparently for the first time, that a number of the common animal and vegetable fats contain small, but easily detectable, amounts of hydroxylated acids.

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FURTHER STUDIES ON THE GROWTH FACTOR REQUIRED BY CHICKS*

THE ESSENTIAL NATURE OF ARGININE

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In a previous paper (1) we reported that chicks fed a ration of yellow corn, wheat middlings, casein, minerals, yeast, and fat-soluble vitamins respond to a growth-promoting factor present in water-extracted liver residue. The insolubility and stability of this factor seem to distinguish it from the active growth factors obtained from liver by other workers (2, 3). The inability of yeast to exert a growth-promoting effect similar to that obtained with liver distinguishes our factor from the one reported by Hogan and Boucher (4).

The fact that the growth factor became water-soluble after hydrolysis of the liver residue with acid suggested that the active substance could be an amino acid. In this paper we wish to present further studies on the distribution, isolation, and identification of the active factor which we originally found in liver residue. The activity of pure *D*-arginine has been demonstrated through the use of two distinctly different rations. Although arginine is not considered to be an essential amino acid in nutrition, the increased rate of growth produced in chicks when the rations used in our studies were supplemented with this amino acid demonstrates that arginine has a profound effect on the metabolism of growing chickens.

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EXPERIMENTAL

Since we had encountered considerable difficulty in attempts to isolate the growth factor from liver residue, we made an effort to find more concentrated sources of the active factor. White Leghorn chicks were used exclusively as experimental animals. Day-old chicks with an initial weight of about 35 gm. were divided into groups of ten and placed in pens equipped with wire screen bottoms and suitable warmers. Weights were recorded weekly. Ration 351 used in our previous studies (5) was used as the basal ration. It has the following composition.

Yellow corn.....	58	Yeast.....	1
Standard wheat middlings..	25	Pptd. CaCO_3	1
Crude domestic casein.....	12	“ $\text{Ca}_3(\text{PO}_4)_2$	1
NaCl.....	1	Cod liver oil.....	1

Previous feeding trials have shown that the average weight at 6 weeks of a large number of chicks on the above ration was 334 gm. When the ration was supplemented with 10 per cent water-extracted liver residue,¹ the average weight was 455 gm., or an increase of 121 gm. Other food materials were added to the basal ration to determine whether a greater growth response could be obtained than that observed with liver residue. Most of the materials assayed were animal tissues, although yeast, wheat germ, and soy bean meal were included as plant sources of the factor. Carefully prepared precipitated skim milk casein was also included to determine whether the activity of casein might be decreased owing to the commercial methods of manufacture. The results are given in Table I. It is readily seen that none of the materials tested surpassed the activity of liver residue. Pork heart and soy bean meal showed activity approaching that of liver residue. The other animal tissues as well as wheat germ were somewhat less potent. A new sample of Yeast III had little activity, although it was obtained from the same source as the one which gave a good growth response previously. This demonstrates that yeast may vary somewhat but that the majority of the samples show no potency. The inactivity of the

¹ The liver residue used in this work was supplied by Dr. David Klein of The Wilson Laboratories, Chicago.

precipitated skim milk casein shows that less severe treatment of the casein does not improve its growth-promoting property. Thus we were unable to find materials more promising than liver residue to serve as a source of the growth factor.

Fractionation of Whole Liver—Since hydrolysis of liver residue with pepsin, trypsin, or acids did not give complete solution of the active factor, attempts were made to liberate the factor by autolysis.

TABLE I

Increased Growth of Chicks on Ration 351 Supplemented with Various Food Materials

Experiment No.	No. of chicks	Supplement to Ration 351	Increase over weight on basal ration at 6 wks.
		<i>per cent</i>	<i>gm.</i>
49	5	12 pptd. skim milk casein	22
33	10	10 Yeast III	34
37	6	10 " III	29
42	6	15 beef spleen*	43
43	10	10 ox lips*	43
37	8	10 pork brain*	52
33	9	15 wheat germ†	61
37	7	10 pork muscle*	64
45	8	15 " spleen*	65
37	6	10 " kidney*	72
49	6	15 soy bean meal	59
48	5	15 " " "	114
39	8	10 pork heart*	83
35	8	10 " "	113

* The vacuum-dried animal tissues were supplied by L. M. Tolman of Wilson and Company, Chicago.

† The wheat germ was supplied by General Mills, Inc., Minneapolis.

20 pounds of ground fresh liver were added to 7.5 gallons of 0.1 N hydrochloric acid and 500 cc. of chloroform. The liver was incubated at 37° for 10 days. At the end of this time, the supernatant liquid was siphoned off. The residue was washed with 5 liters of water. After the supernatant liquid was decanted, the insoluble material was centrifuged off and dried for assay. The supernatant liquid and washings were combined and concentrated by distillation under reduced pressure. The syrupy

concentrate was poured on a known amount of basal ration and dried in the drying room at 50°.

Assays of the filtrate and residue after autolysis are shown in Table II, Experiment 32. The inactivity of the filtrate is further evidence that our growth factor is distinct from physins described by Mapson (3).

TABLE II

Growth of Chicks on Ration 351 Alone and on Basal Ration Supplemented with Liver Residue or with Fractions from Whole Fresh Liver

Experiment No.	No. of chicks	Supplement to Ration 351	Weight at 6 wks.
			gm.
32	8	None	316
	7	Autolyzed liver filtrate \approx 12% liver	351
	8	“ “ residue \approx 12% “	430
36	7	None	310
	7	10% liver residue	405
	8	NaOH-extracted residue \approx 12% liver	333
	7	NaOH-soluble material \approx 12% “	437
	8	HCl ppt. of NaOH-soluble material \approx 12% liver	391
	8	Filtrate after HCl pptn. \approx 12% liver	358
44	8	None	333
	7	10% liver residue	448
	6	Acetone, ether, 70% alcohol-extracted HCl ppt.	417
	8	70% alcoholic extract of HCl ppt.	348
47	7	None	376
	7	10% liver residue	431
	6	6% edestin	458
	7	15% peanut meal	558

Since the treatment of liver with enzymes did not yield ready solution, we were led to the use of dilute alkali to dissolve the factor.

Finely ground fresh liver was treated with 95 per cent alcohol. Sufficient alcohol was added to make the final concentration 50 per cent. The solids were readily filtered off with suction. The residue was stirred into 15 liters of distilled water. After mixing to disperse any large clumps, a 20 per cent solution of sodium hydroxide was added until the concentration of alkali reached 0.3 per cent (pH 8.0 with cresol red).

After 2 hours, the insoluble material was removed by filtration through cheese-cloth. The filtrate was divided into two equal parts. One portion was adjusted to pH 7.0 (brom-thymol blue) and evaporated to dryness. The second portion was diluted with an equal volume of distilled water, acidified to pH 5.8 (brom-cresol purple) with hydrochloric acid, and allowed to settle overnight. The light tan precipitate was filtered off and dried for assay. The nitrogen content of the dried precipitate was 11.01 per cent (protein = 68.8 per cent). The filtrate was adjusted to pH 7.0 and evaporated to dryness for assay. The distribution of the growth factor after alkali treatment is shown in Table II, Experiment 36.

It is definitely evident that the entire activity of the liver was recovered in the alkali-soluble fraction and that the residue showed no potency. Acidification of the alkali solution to pH 5.8 precipitated the growth factor along with the bulk of the protein material.

In order to determine whether the activity was adsorbed or occluded by the voluminous precipitate that resulted when acid was added to the alkaline solution, the following experiment was performed. The material precipitated by acid at pH 5.8 after solution in 0.3 per cent alkali was extracted successively with acetone, ether, and 70 per cent alcohol. The results secured are shown in Table II, Experiment 44. The activity definitely remained in the residue after acetone, ether, and alcohol extraction and confirmed the association of the growth factor with the protein fraction.

Identification of Arginine As the Growth Factor—Since liver proteins are known to be rich in basic amino acids, a suggestion by one of us (C. A. E.) led us to try arginine. Two materials, peanut meal and edestin, were selected for use in the preliminary trials.

Edestin was prepared by removing the fat from hemp-seed^a with ether and then extracting with 5 per cent sodium chloride at 60°. After chilling and filtering, the edestin was recrystallized. The final product gave only a faint test for phosphorus. Edestin contains 15.8 per cent arginine (6). Fresh unroasted peanuts

^a The hemp-seed used in this work was supplied by Dr. J. W. Hayward of the Archer-Daniels-Midland Company, Milwaukee.

were hulled, dried, and extracted with ether. The ether removed 47 to 52 per cent of the dry weight. The remaining material was mainly protein, and arachin, the main protein of peanuts, has 12.5 per cent arginine (7). Since a Van Slyke determination of the arginine in liver gave a value of 6 per cent of arginine in dried whole liver, the peanut meal and edestin supplements to Ration 351 were made so that each furnished about the same amount of arginine. The liver residue supplement incorporated into Ration 351 at a 10 per cent level (equivalent to 12 per cent whole liver) added 0.72 per cent arginine to the ration. Edestin added at a 6 per cent level and peanut meal at a 15 per cent level to Ration 351 contributed about 0.9 per cent arginine to Ration 351. Feeding trials with these sources of arginine gave the results shown in Table II, Experiment 47.

The remarkable stimulation obtained with edestin and peanut meal led us to repeat and extend our experiments. Arginine was prepared according to the procedure of Kossel and Gross (8). Decomposition of the flavianate was conducted at room temperature with barium hydroxide in slight excess without the use of ammonia. The last traces of color after filtering off the barium flavianate were readily removed with charcoal at room temperature. In order to eliminate the possibility that the positive effect of peanut meal could be due to added vitamin B₄, peanut meal was heated for 24 hours at 120° to destroy any residual vitamin B₄ according to the procedure of Keenan, Kline, Elvehjem, and Hart (9).

Ration 351 was fed for a negative control. The positive control liver residue consisted of Ration 351 plus 10 per cent liver residue. The supplements were fed at the following levels: peanut meal, both heated and unheated, at a 15 per cent level, edestin at 6 per cent, and arginine carbonate or hydrochloride at 1 per cent. The arginine carbonate contained 74 per cent arginine and the arginine hydrochloride 82 per cent arginine. It has been necessary to repeat these trials several times, since our results have been complicated by gizzard lesions. The presence of gizzard lesions has retarded growth, especially in the first 3 weeks of the experiment. Chicks from different sources have displayed the same irregularity of performance. In spite of this

difficulty the data in Table III consistently show the supplementary effect of arginine-rich materials as well as arginine itself. The addition of less than 1 per cent of arginine as the crystalline salt or from two distinctly different source materials provided growth in chicks approximately equal to that obtained with liver residue additions.

TABLE III
Growth-Promoting Property of Arginine and Arginine-Rich Materials

Experiment No.	Supplement to Ration 351													
	Basal Ration 351 only		10% liver residue		15% peanut meal		15% heated peanut meal		6% edestin		1% arginine carbonate		1% arginine hydrochloride	
	No. of chicks	Weight, 6 wks.	No. of chicks	Weight, 6 wks.	No. of chicks	Weight, 6 wks.	No. of chicks	Weight, 6 wks.	No. of chicks	Weight, 6 wks.	No. of chicks	Weight, 6 wks.	No. of chicks	Weight, 6 wks.
		gm.		gm.		gm.		gm.		gm.		gm.		gm.
48	3	330	7	419	5	407	6	342			4	410		
49	4	301	5	392	4	389	6	375	6	398	4	404		
50	10	343	9	371	8	356	7	380	7	354	8	371		
52	8	342	10	406	8	376	9	384	6	388			7	395
Total.....	25		31		25		28		19		16		7	
Average..		334		396		379		372		379		389		395

The growth-promoting effect of arginine has been verified with Ration 452 (10). This ration has the following composition.

Dextrin.....	64	Crude liver extract.....	2
Purified casein.....	18	Autoclaved liver residue....	2
Water-extracted lung.....	5	Brewers' yeast.....	2
Salts I (10).....	5	Cod liver oil.....	2

This ration, used for vitamin B₄ studies, is also low in the growth factor. To supply vitamin B₄, 10 per cent peanut oil was added to the ration, replacing an equivalent quantity of dextrin. The addition of 1 per cent of arginine hydrochloride yielded the results shown in Chart I. Growth of chicks on Ration 452 supplemented with peanut oil is invariably low and has been observed many times (unpublished results). The addition of peanut meal

or arginine itself to Ration 452 produces excellent chicks, both in appearance and in weight. In this particular instance the addition of 1 per cent arginine hydrochloride increased the growth by approximately 43 per cent.

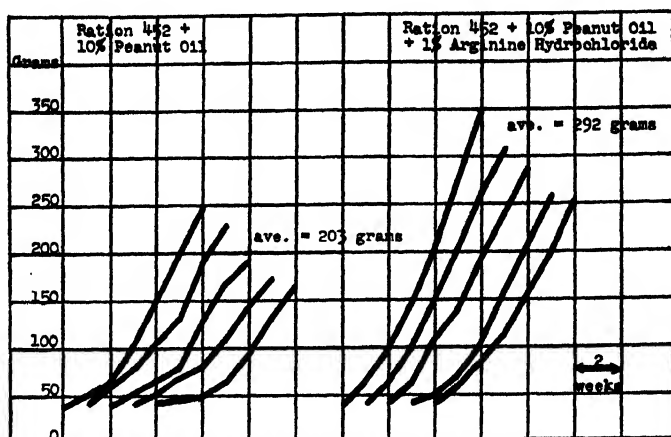


CHART I. Individual growth records of chicks on basal Ration 452 + 10 per cent peanut oil and on basal Ration 452 + 10 per cent peanut oil + 1 per cent arginine hydrochloride.

DISCUSSION

The growth-promoting action of animal organs in the diet has long been recognized. We are able, as a result of these studies with chicks, to show that with a natural ration in which corn and wheat proteins are fortified with casein or where casein is the principal protein the growth rate can be definitely improved by the addition of arginine.

This finding is particularly striking in view of the fact that both basal rations used in our studies contain considerable amounts of arginine. Hart (11) reported 4.8 per cent of arginine in casein. The arginine added to Ration 351 by 12 per cent of crude casein is about 0.57 per cent. The addition of 1 per cent of arginine carbonate or hydrochloride does little more than add an equivalent amount of arginine. Ration 452 contains 18 per cent of casein, which added 0.86 per cent of arginine.

The fact which we have previously reported (1) and which is

confirmed in Table I, that additional amounts of casein in Ration 351 are without effect, indicates that the arginine in casein is not as effective as the arginine from other sources. The reason for this is not clear. The physical state of the casein may be ruled out as a factor, since the casein used in the experiment reported in Table I was precipitated from skim milk with hydrochloric acid, filtered, and dried on a known amount of the grain component of Ration 351. Wide dispersion and mild drying did not improve the value of casein as a source of arginine.

Ration 452 contained 18 per cent of casein or 50 per cent more than was present in Ration 351 but the addition of 1 per cent of arginine hydrochloride resulted in chicks that were over 40 per cent heavier than those on the basal ration.

Incomplete availability of the arginine of casein for chicks seems a likely factor. It is entirely possible that during the rapidly growing period the chick is not capable of digesting certain proteins rapidly enough to meet the demands for arginine. A complementary action by other amino acids may also serve to produce full utilization in such sources as edestin, peanut meal, pork heart, and liver.

Other workers have also reported growth-promoting properties for arginine. Patch (12) noted that the addition of arginine along with cystine to a casein-milk powder diet caused a striking and prolonged increase in erythrocytes, hemoglobin, and growth of *Amblystoma* larvæ. It is to be noted that here also casein did not supply sufficient arginine for dietary needs.

Gudernatsch (13) found that arginine was necessary for the growth of *Rana temporaria* tadpoles, protozoa, and chick tissue cultures. Hammett and Chatalbash (14) reported arginine to have a specific action for accelerating the processes which underlie new growth initiation of *Obelia* hydranths, from which results a secondary enhancement of proliferation.

The results presented in this paper favor the view that arginine is an essential amino acid for chicks and that chicks do not obtain an optimum supply of this amino acid from the ordinary grain rations during the rapid growth period, or that arginine may act by supplementing the ration which may be low in other amino acids. Scull and Rose (15) have shown that rats placed on diets very low in arginine are able to synthesize this amino acid for

medium rates of growth. In the case of the chick we are unable to state whether the synthesis of arginine is more limited or whether greater amounts are needed for the more rapid growth.

The growth-promoting effect of arginine is decreased after 6 weeks with the chick. This heightened activity in the early stages of growth is in good agreement with the results of Brachet and Needham (16) who found large amounts of arginine in the developing chick embryo. One of the mechanisms for the action of arginine has been suggested by Kamachi (17) who has presented evidence to show that arginine injected into developing chick embryos is partly converted into histidine. Experiments designed to disclose the relation of arginine to other amino acids closely related to it chemically are in progress.

SUMMARY

1. Pork heart and soy bean meal are almost as potent as liver residue in stimulating growth in chicks when added to Ration 351. Wheat germ, pork kidney, spleen, muscle, and brain are less active than liver residue. When Ration 351 is supplemented with additional amounts of yeast or casein, no added growth results.

2. Edestin, peanut meal, and liver residue appear to have a growth-promoting action proportional to their arginine content when used to supplement Ration 351.

3. An excellent growth response is obtained with chicks when 1 per cent arginine carbonate or hydrochloride is added to a yellow corn middlings-casein ration (Ration 351) or to a dextrin-casein ration (Ration 452 + 10 per cent peanut oil).

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STUDIES ON THE GLUTATHIONE CONTENT OF THE BLOOD IN NUTRITIONAL ANEMIA*

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In a study of the copper content of the blood in nutritional anemia (1) it was observed that in pigs depleted of their bodily copper stores the copper of the blood falls to extremely low values and that upon feeding sufficient amounts of copper it is restored very rapidly to normal levels. It was suggested then that with a low copper concentration in the blood, the medium surrounding the young blood cells in the bone marrow would not possess the right physical and chemical properties to permit formation of hemoglobin and maturation of erythrocytes. Clinical observations (quoted in (1)) established that in various conditions of anemia, *i.e.* with increased demand for formation of hemoglobin and of erythrocytes, the copper content of the blood increases. Under similar conditions, in hemorrhagic or in phenylhydrazine anemia of rabbits (2-6), and in various forms of human anemia (2, 7, 8), the glutathione content of the erythrocytes was found to be increased. Other considerations pointed also to a possible correlation between the copper content of the blood and the level of reduced and oxidized glutathione in the blood. Tompsett (9) suggested that part of the copper inside the red blood cells may occur as the cuprous mercaptide of glutathione. The oxidation of reduced glutathione is a metallic catalysis (10), probably

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brought about by iron and copper complexes in the blood (11, 12). With these facts in mind we studied the glutathione content of the blood of rats and of pigs under conditions of retarded and accelerated activity of the hematogenic organs.

EXPERIMENTAL

The rats used in this work were made anemic as usual on an exclusive milk diet. When the hemoglobin content of the blood had decreased to 3 to 4 gm. per 100 cc., some of the animals were used for glutathione analyses; others were fed a daily supplement

TABLE I
Glutathione in Rat Blood

Treatment	No. of rats	Hb per 100 cc.		Reduced glutathione		Total glutathione	
		Before	After				
		gm.	gm.	mg. per 100 gm.	mg. per million r.b.c.*	mg. per 100 gm.	mg. per million r.b.c.*
Stock rats, mature.....	7			40.43	5.26	42.39	5.44
“ “ 6 wks. old.....	8			44.45			
Anemic.....	4	5.50		5.13	1.72		
“	7	4.11				16.68	4.71
“	7	3.68		6.28	2.08		
0.5 mg. Fe for 5 days.....	7	3.41	3.59	1.40	0.50		
0.1 “ Cu “ 5 “	7	2.95	3.84	6.23	1.78		
0.5 “ Fe + 0.1 mg. Cu for 5 days.....	7	3.17	7.83	28.69	6.24		

* Mg. glutathione in 100 gm. blood

Millions red blood cells in 1 c.mm. blood

of 0.5 mg. of Fe, or 0.1 mg. of Cu, or 0.5 mg. of Fe + 0.1 mg. of Cu for a period of 5 days. At the end of this period, *i.e.* at a time when the rat shows a maximum reticulocyte response if both iron and copper are fed (13), the blood was analyzed for glutathione. The blood was withdrawn from the abdominal aorta by the procedure of Swanson and Smith (14). It was discharged into a weighed test-tube containing 5 cc. of redistilled water with oxalate as anticoagulant. After weighing, sufficient water was added to bring the proportion of blood to water to 1:8. Glutathione was determined by the method of Woodward and Fry

(15). Young, anemic rats usually do not yield enough blood to permit satisfactory analyses of both reduced and total glutathione on the same sample. The values reported in Table I were obtained from different rats.

The glutathione analyses of pig blood were made by the same method as in our studies on the copper content of the blood of anemic pigs (Pigs 20 to 27, (1)).

RESULTS AND DISCUSSION

Because the glutathione of the blood is carried only in the erythrocytes (2, 16-18), the results are expressed in terms of glutathione per million erythrocytes. In the case of rat blood we also report mg. of glutathione per 100 cc. of blood.

Table I summarizes the results obtained with rat blood. It is evident that with development of nutritional anemia the reduced glutathione content of the red cells fell to low levels. At this point it was not raised by feeding either iron alone or copper alone. But when both iron and copper were fed, *i.e.* during rapid recovery from nutritional anemia, there was a rapid rise of the reduced glutathione content of the erythrocytes to normal values. Quite unexpected was the observation that in nutritional anemia of rats there is a marked shift in the proportion of reduced to oxidized glutathione. Normally the reduced form accounts for 90 to 100 per cent of the total glutathione of the erythrocytes; in nutritional anemia of rats it is less than 50 per cent. Feeding iron alone tended further to depress the amount of reduced glutathione in the erythrocytes. Upon feeding both iron and copper, however, the equilibrium is apparently rapidly shifted back to the normal proportions. Three rats getting both iron and copper yielded enough blood to permit analysis for both forms of glutathione, the reduced form accounting for from 86 to 90 per cent of the total. The present knowledge of blood chemistry cannot explain the significance of these observations. It is a matter of conjecture whether the increase of total glutathione in the red cells and the shift from the oxidized to the reduced form after feeding of both iron and copper is a direct effect of the two metals on glutathione or whether it is secondary to rapid formation of hemoglobin and erythrocytes. Litarczek *et al.* (19) have suggested that the increased glutathione content of the erythro-

cytes following hemorrhage in rabbits is due to the increased number of reticulocytes which contain more glutathione than the mature cells. This would tend to correlate hematogenic activity of the bone marrow with the glutathione content of the red cells. It might be argued that increased glutathione content is due to the high reticulocyte count; however, in normal animals with a low number of reticulocytes, the glutathione content of the blood is the same.

TABLE II

Average Glutathione Content of Pig Blood at Various Stages of Nutritional Anemia and Recovery

The results are expressed as (mg. of glutathione in 100 cc. of blood)/(millions of red blood cells in 1 c.mm. of blood).

Hb per 100 cc. blood	Milk; no metals			Milk + Fe			Milk + Fe + Cu		
	Reduced	Total	Per cent oxidized	Reduced	Total	Per cent oxidized	Re- duced	Total	Per cent oxi- dized
<i>gm.</i>									
10-12							4.35	4.73	8.0
9-10							3.76	4.13	8.9
8- 9							3.64	4.64	23.7
7- 8	4.76	5.85	18.6	4.20	4.67	10.0	4.96	5.95	16.6
6- 7	3.74	4.33	13.6	5.23	5.60	4.8	4.88	5.88	17.0
5- 6	4.86	5.22	16.3	6.27	6.62	5.3	5.79	5.81	0.3
4- 5	5.62	6.51	13.6	7.17	8.21	12.7			
3- 4	6.42	6.92	7.2	7.23	8.38	13.7			
2- 3	7.20	8.25	12.7	7.30	9.07	19.5			

The results obtained with pig blood, summarized in Table II, are entirely different. Although we observed considerable fluctuation in the glutathione content of the erythrocytes in successive bleedings, it is evident that in pigs the glutathione content of the cells increased with progressive anemia and that it returned to lower values during recovery. No constant shift in the proportion of the two forms of glutathione could be observed. This is directly opposite to the results obtained with rat blood, but it conforms with the observations recorded in the literature to which we referred. It appears that in pig blood the glutathione content of the erythrocytes is dependent primarily upon the degree of

anemia of the animals. It was roughly the same in animals having about the same hemoglobin content of the blood, irrespective of their dietary intake of iron and of copper.

Iodometric titrations of blood and of tissue filtrates are not specific for glutathione. Substances known to reduce iodine besides glutathione are ascorbic acid, cysteine, and thioneine. Ascorbic acid is present in the blood at least partly in the oxidized form (20) and would therefore not interfere with the determination of GSH. It has also been shown that the ascorbic acid content of the blood depends upon dietary intake (21), which would be not very high on an exclusive milk diet. The same applies to thioneine because Potter and Franke (22) have found that the thioneine content of the blood of rats on a diet containing 20 per cent commercial, crude casein and 10 per cent butter fat was less than 0.1 mg. per 100 cc. of blood. Particularly important is the observation of Quensel and Wachholder (23) that upon destruction by formaldehyde of GSH in blood filtrates according to Woodward and Fry, the reducing value of the filtrate is lost. This conforms with the work of Woodward (24) who showed that in the determination of glutathione by glyoxalase activation, thioneine, cysteine, and ascorbic acid are not included and that the reduced glutathione determined in this manner agrees with the values obtained by iodine titration.

We cannot offer any explanation for the observed differences in the two species. It must be borne in mind, however, that although the reducing value toward iodine of blood filtrates is apparently due mostly to glutathione, the possibility exists that during anemia of pigs and perhaps other animals, reducing substances other than glutathione may appear in the blood and thus account for the increased iodine titrations.

SUMMARY

1. In rats suffering from nutritional anemia the total glutathione content of the erythrocytes is decreased.
2. At the same time there is a marked shift towards the oxidized form of glutathione, causing a great decrease in the amount of reduced glutathione in the erythrocytes.
3. During recovery from anemia as a result of feeding of iron and copper the normal conditions are rapidly restored.

4. In nutritional anemia of pigs there is an increase of both the total and the reduced glutathione content of the erythrocytes as the anemia becomes severe.

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VITAMIN C IN VEGETABLES

IV. ASCORBIC ACID OXIDASE*

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The oxidation of ascorbic acid to dehydroascorbic acid may be catalyzed by copper (1, 8), by other metals (11) and metallic compounds, by norit charcoal (5), and by an enzyme or enzyme complex (12, 13). The dehydroascorbic acid has an antiscorbutic activity similar to that of ascorbic acid (6) and may be reduced to the latter by the use of H_2S and other reductants (14). The existence of ascorbic acid oxidase in cabbage leaves was shown by Szent-Györgyi (12) in 1931. Tauber, Kleiner, and Mishkind (13) prepared from Hubbard squash a highly active enzyme which is claimed to be specific for ascorbic acid inasmuch as it does not catalyze the oxidation of cysteine, tyrosine, glutathione, and phenols. The ascorbic acid may further change into a compound which cannot be reduced to ascorbic acid and which is inactive as an antiscorbutic agent. According to Borsook and Jeffreys (3), this compound, yet unidentified, is not an oxidation product.

The present paper deals with the enzyme complex participating in the oxidation of ascorbic acid in spinach, beans, peas, pumpkin, turnips, and cabbage. Results of experiments dealing with the thermal inactivation of the enzyme are presented and the practical importance of the enzyme from the standpoint of nutrition and food preservation is discussed.

EXPERIMENTAL

During the extraction of minced vegetables with water much of the ascorbic acid present is oxidized into dehydroascorbic acid (4)

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which is physiologically active but is not detected by the indophenol dye. In water extracts of vegetables like cabbage, sweet corn, parsnips, pumpkin, and squash, which contain a very active enzyme, the ascorbic acid is entirely oxidized during the extraction period of 10 to 15 minutes, but can be completely recovered by immediate treatment with H_2S followed by elimination of the excess H_2S by CO_2 . In the case of other vegetables, such as string beans and Lima beans, peas, Swiss chard, carrots, and especially spinach, the enzymic oxidation is much slower and a considerable portion of the ascorbic acid may be extracted with water in the original form. Heating for a period sufficient to inactivate the oxidase has a beneficial effect on the preservation of ascorbic acid in vegetables, but it does not prevent losses caused by other catalysts. The extent of loss by non-enzymic catalysis varies considerably in different vegetables.

Our main interest being in the practical significance of the enzyme action rather than in a study of the enzyme itself, all the work reported here was performed on vegetable extracts which were used without further attempts at purification. It was of considerable importance to establish the extent of heating needed for the inactivation of the ascorbic acid oxidase in vegetables. To exclude the great differences in the rate of heat penetration, this was done under uniform conditions. The vegetable was ground with an equal weight of water and 5 cc. of this mixture were immersed in boiling water for a definite time interval. After cooling, 1 cc. of a solution containing 0.5 mg. of ascorbic acid was added and the mixture kept at 30° for 3 hours. Changes in the ascorbic acid content were followed by titrating the mixture by the usual method with the indophenol dye. The results obtained are shown in Fig. 1. For reasons to be discussed later, this figure also shows the relative catalase activity of the similarly heated samples as determined at pH 7 by the usual method, the residual hydrogen peroxide being estimated after 1 hour.

It appears from Fig. 1 that the ascorbic acid oxidase was completely inactivated in all cases in 1 minute, and no further changes in the enzyme activity were caused by prolonged heating. The loss caused by non-enzymic catalysis is considerable during the 3 hour period, and different blanks must be run for every period of heating. It was found that the enzymic catalysis of the oxida-

tion is rather slow in spinach and that the non-enzymic catalysis of the oxidation was more rapid than in any of the other vegetables studied. This fact might be attributed to the high iron and copper content of spinach. In Fig. 2 the results obtained with a spinach extract are compared with those obtained with a cabbage extract. The oxidation caused solely by the enzyme action may be calculated by subtracting the value for the heated from that of the

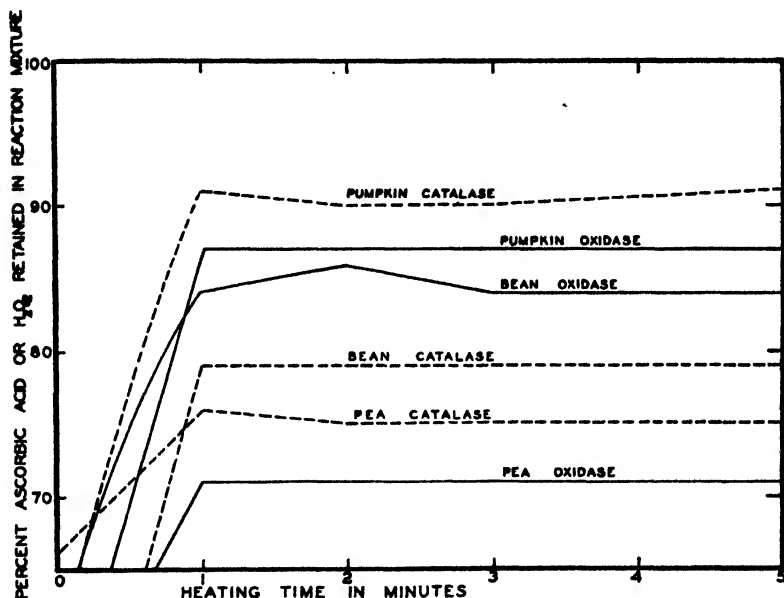


FIG. 1. Inactivation of ascorbic acid oxidase and catalase in vegetable extracts at 100°.

unheated extracts. It is apparent that the enzymic oxidation in spinach is only a fraction of that observed in the cabbage extract.

In spinach and beans cooked in boiling water for 1 minute the ascorbic acid oxidase was completely inactivated. Peas require from 30 to 60 seconds of heating in boiling water or steam to obtain a blanch sufficient to preserve desirable characteristics when the peas are to be frozen. The most desirable duration of scalding is usually established by a determination of the catalase in the vegetable. For best results no catalase activity should be present in an extract of the blanched vegetable. It was of importance

to know, therefore, the relation between the rate of heat inactivation of catalase and ascorbic acid oxidase.

It was shown in Fig 1 that there is a good correlation between the inactivation of ascorbic acid oxidase and catalase in extracts of pumpkins, beans, and peas. In Table I similar information is presented on whole peas heated for different lengths of time. In all cases, both the ascorbic acid oxidase and the catalase were

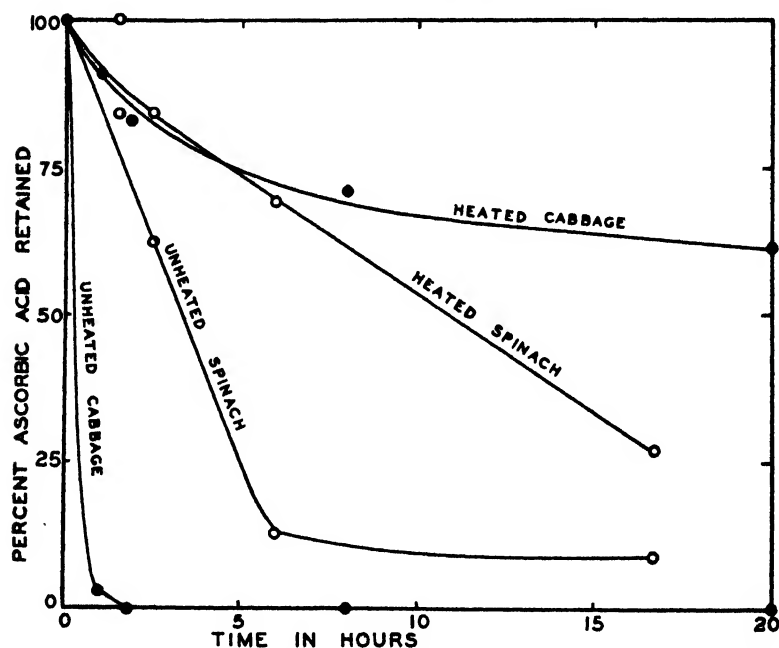


FIG. 2. Oxidation of ascorbic acid in raw and heated cabbage and spinach extracts.

completely inactivated by 1 minute of heating, while half a minute of heating gave a complete inactivation of the ascorbic acid oxidase in the steam-blanching peas only. The heating required to inactivate the catalase also inactivates the ascorbic acid oxidase.

To test the influence of the inactivation of ascorbic acid oxidase on the preservation of ascorbic acid, vined (machine-shelled) Thomas Laxton peas, blanched in steam for 30, 60, 120, and 300

seconds, were quick-frozen in a Birds Eye multiplate freezer and stored at -7° for 2 months. The ascorbic acid content of the samples was then determined by a modified chemical method (10). The results of these determinations are given in Fig. 3. In agreement with the results of enzyme determinations, the maximum retention of ascorbic acid was reached by 1 minute of heating, which was sufficient for the complete inactivation of both ascorbic acid oxidase and catalase. Attempts to regenerate the lost ascorbic acid by treatment with H_2S failed except in the unblanched sample in which case there was a nominal increase.

TABLE I
Heat Inactivation of Ascorbic Acid Oxidase and Catalase in Thomas Laxton Peas

Duration and temperature of blanching	Relative enzyme activity	
	Ascorbic acid oxidase (per cent of added ascorbic acid lost in 3 hrs. at 30°)	Catalase (per cent of added H_2O_2 lost in 1 hr. at 30° , pH 6.8)
No treatment.....	54	74
0.5 min. at 82°	23	35
2 " " ".....	17	39
5 " " ".....	17	
0.5 " with steam.....	13	73
1 " " ".....	14	37
2 " " ".....	12	36
5 " " ".....	14	42

Dehydroascorbic acid may be completely reduced to ascorbic acid if the treatment is performed without delay. After a certain time, however, the length of which depends on the material and on the experimental conditions, the dehydroascorbic acid is further transformed into a compound or compounds which are biologically inactive and cannot be reduced to ascorbic acid in the usual way. In an experiment on cabbage, the results of which are presented in Table II, the different steps of decomposition of ascorbic acid in the presence and absence of the oxidase are shown. An aqueous extract of 20 gm. of cabbage per 100 cc. was prepared. A part of this extract was heated to 100° for 3 minutes and cooled. 5 mg.

of ascorbic acid solution were added to 50 cc. portions of the heated and unheated extracts. At given time intervals an aliquot of this mixture was titrated with the indophenol dye. The "regeneration" was performed by bubbling H_2S through the solution for 10 minutes, letting the sample stand in the corked test-tube for 1 hour, and bubbling CO_2 through the solution for 2 hours or until the test, as proposed by Johnson and Zilva (7), for H_2S was

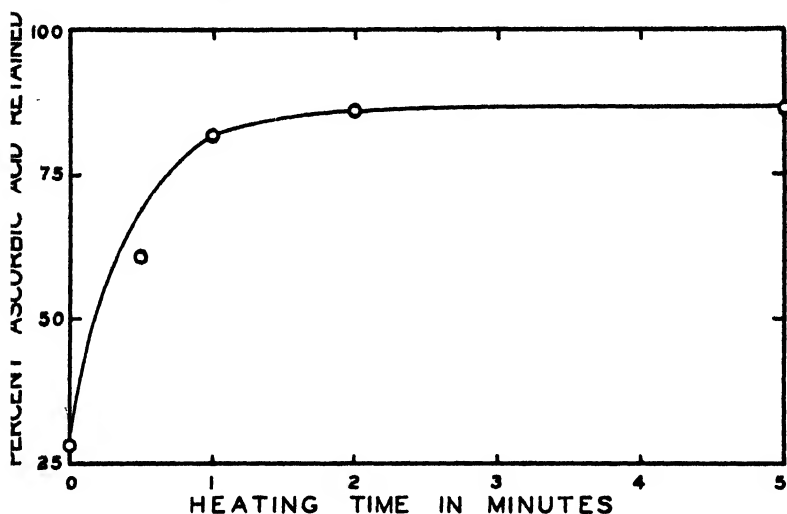


FIG. 3. Retention of ascorbic acid in raw and heated peas frozen at -33° and stored at -7° .

negative. The irreversible reaction product in the fourth and seventh columns of Table II was determined by difference.

The irreversible reaction at pH 3.7 is similar to that occurring in a more nearly neutral solution, and non-antiscorbutic products are formed in both cases (2). This non-antiscorbutic reaction product reduces the indophenol dye in neutral or faintly acid solution. In our experiments the titration was performed in 6 per cent acetic acid, so that the pH of the solution was about 3.0. It is believed that the reaction between the dye and the irreversible form of the first oxidation product of ascorbic acid is not appreciable in strongly acid solution. Hence, this method of analysis for the three forms of ascorbic acid should be valid.

The ascorbic acid disappeared rapidly from the solution in the presence of the enzyme. The decomposition of the ascorbic acid from non-enzymic causes was relatively much slower, only about 17 per cent in 3 hours. There also appears to be a correlation between the proportion of ascorbic acid finally lost for antiscorbutic purposes and enzyme action inasmuch as the loss is much more rapid in the presence of the enzyme.

TABLE II

Decomposition of Ascorbic Acid in Cabbage Extract in Presence and Absence of Ascorbic Acid Oxidase

The results represent the percentage of the initial ascorbic acid content. The initial pH of all the reaction mixtures was 3.7.

Time <i>hrs.</i>	With enzyme (not heated)			Without enzyme (heated)		
	Ascorbic acid	Dehydro-ascorbic acid (regenerable)	Decomposed (not regenerable)*	Ascorbic acid	Dehydro-ascorbic acid (regenerable)	Decomposed (not regenerable)*
0	100	0	0	100	0	0
1	3	88	9	91	4	5
3	0	79	21	83	2	15
20	0	40	60	61	15	24

* Physiologically inactive.

DISCUSSION

The fact that many vegetables contain an active ascorbic acid oxidase was to be expected from reference to the literature. It was also expected that this oxidase would be relatively easily inactivated by heating. The enzyme is interesting from the standpoint of the plant biochemist because unquestionably much significance should be attached to the presence and rôle of ascorbic acid and ascorbic acid oxidase in plants. It is considered, however, that ascorbic acid oxidase catalyzes the formation of dehydroascorbic acid, an antiscorbutic agent equal in effectiveness to ascorbic acid; the nutritionist might question the practical significance of the presence of this enzyme in plants.

The authors believe that evidence presented in this paper, as well as several observations previously recorded in the literature, points to the great importance of this enzyme in the preservation

of vegetables. An inspection of Table II shows that at any time the proportion of the dehydroascorbic acid in the mixtures was much larger in the presence than in the absence of the enzyme. By assuming that the rate of decomposition of dehydroascorbic acid is a function of its concentration, one obtains a reasonable explanation of the more rapid loss of physiologically active forms of ascorbic acid in the presence of the enzyme. The rate of decomposition of dehydroascorbic acid, as shown in Table II, is considerably greater than that reported by Wurmser and Sonbeiro (15). We have found that the irreversible decomposition of ascorbic acid in vegetable extracts, even with the enzyme inactivated, is more rapid than in pure aqueous solutions of ascorbic acid of the same concentration and pH.

Further evidence supporting this theory can be found in Fig. 3 in which the ascorbic acid content of raw and blanched peas frozen and stored at -7° is shown. The highest loss of ascorbic acid occurred in the unblanched sample in which, after 2 months of storage, a small proportion of the original ascorbic acid was found in the form of dehydroascorbic acid. The loss of ascorbic acid was less in the sample blanched for half a minute, and in the samples blanched for 1 minute or more no loss was observed except the uniform slow non-enzymic degradation. Again the indirect detrimental effect of the enzyme on the preservation of the ascorbic acid is indicated.

From the large number of cases which could be cited in support of this hypothesis only one will be mentioned here. As early as 1931, Kohman, Eddy, and Gurin (9) reported that minced carrots lost much of their antiscorbutic activity upon standing in air for 1 hour. At the time of these investigations dehydroascorbic acid was unknown. Later work revealed that heating (blanching) practically prevented this loss of ascorbic acid in minced vegetable tissues. The loss of ascorbic acid in a physiologically active form was more rapid where the ascorbic acid had been transformed by enzyme action into dehydroascorbic acid.

SUMMARY

1. Enzymes participating in the oxidation of ascorbic acid have been shown to be generally present in vegetables. Already known to be present in cabbage and squash, it has also been found in

pumpkins, peas, string beans, Lima beans, sweet corn, Swiss chard, carrots, parsnips, and spinach. The relative activity of this enzyme varies greatly in different vegetables.

2. Ascorbic acid oxidase is completely inactivated in vegetables or in their extracts by heating to 100° for 1 minute.

3. The presence of ascorbic acid oxidase is instrumental in the loss of physiologically active forms of ascorbic acid by catalyzing the transformation of this latter into dehydroascorbic acid, which is more readily decomposed by a non-enzymic reaction into a compound having no antiscorbutic activity.

4. Peas frozen after sufficient heat treatment to inactivate the ascorbic acid oxidase retained a much greater portion of their original ascorbic acid content than those having the active enzyme present. Since the ascorbic acid oxidase and catalase appear to be inactivated by heat at the same rate, the commercial practice of establishing the proper blanching time by a test for catalase activity also yields information on the inactivation of the ascorbic acid oxidase.

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THE MOLECULAR WEIGHT OF INULIN*

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Previous estimations of the molecular weight of inulin have yielded results which vary from 164,000 to 162. Most reliable appear to be the values obtained by Irvine and coworkers (4700 to 4900) (1) based upon methylation, those of Haworth and coworkers (3600 to 5200) (2) based on methylation, acetylation, boiling point and freezing point changes, and those of Berner (3300 to 5100) (3) based on the two latter methods. Recent studies (4) on the mode and rate of the renal excretion of intravenously injected inulin have a theoretical and practical significance which depends largely on the correctness of our information concerning the molecular weight and volume of this substance. The diversity of the estimations cited above is so great that further study seemed desirable. This fact, together with the availability of a peculiarly suitable method, not hitherto used in studying inulin, led to the work here described.

Method

The osmotic pressure of inulin in aqueous solution was determined by the thermoelectric vapor pressure method developed by Hill (5) as modified by Baldes (6). For a complete description of apparatus and technique the reader is referred to the latter paper. In brief, the apparatus is composed essentially of two (duplicate) thermocouples, each consisting of two opposed constantan-manganin thermal junctions in the form of minute wire loops appropriately connected with a highly sensitive galvanometer.

* The expenses of this investigation have been defrayed in large part from a grant by the Commonwealth Fund.

The inulin solution was placed on one loop of each pair, distilled water on the opposite loop of each pair. The thermocouples were then screwed into a small air-tight chamber, the inner surface of which was covered with filter paper thoroughly saturated with distilled water. The chamber was immersed in a well stirred water bath maintained approximately at room temperature (21.7–23.3°). A sensitive thermostatic control mechanism kept the temperature of the water bath constant within 0.001° throughout an observation. Within 5 to 10 minutes after the chamber was closed and immersed in the bath the difference in the vapor pressure of the two solutions (*i.e.*, inulin solution *versus* distilled water) produced a persisting minute difference in the temperatures of the two loops of each thermocouple. This difference was measured by recording repeatedly the deflection of the galvanometer during the succeeding 20 minutes.

Since the method does not give absolute values, a reference standard is required; for this purpose aqueous sodium chloride solutions, from 0.05 to 0.11 per cent, were used. The effective molality for these solutions was calculated from data given in the International Critical Tables. To correct for drift which might conceivably occur during the course of the inulin observations, the deflection produced by a known solution of sodium chloride *versus* water was measured at the beginning and end of each series. To correct for asymmetry in the apparatus, each solution was reversed with respect to its position on the loops of each thermocouple, the finally recorded figure for galvanometric deflection being the average of four single determinations. To check the accuracy of the apparatus and procedure as a whole, each determination of the molecular weight of inulin was preceded by a control estimation of the molecular weight of sucrose.

One complete experiment included therefore (1) two or more calibrating determinations with NaCl *versus* distilled water, (2) one or more determinations with a sucrose solution *versus* distilled water, so that a known molecular weight might be calculated as a control, and (3) one to four determinations with a solution of inulin, the molecular weight of which was to be determined. The actual concentrations of NaCl, sucrose, and inulin solutions were unknown to the individual who carried out the vapor pressure measurements.

Baldes (6) found the error of the method to be ± 0.2 per cent for solutions isotonic with blood, indicating that it was not capable of distinguishing solutions differing by less than the osmotic equivalent of 4 mg. of NaCl per 100 gm. of water. This was confirmed for our apparatus by comparing 0.9 and 1.0 per cent NaCl solutions. The high molecular weight and low solubility of inulin (at 20–22°) required working with concentrations osmotically equivalent to between 0.12 and 0.06 per cent NaCl. At such levels, lying considerably nearer to the limit of sensitivity, the error, as would be expected, was greater—as much as ± 3 to ± 8 per cent.

For correcting the apparent weight of inulin to the true mass *in vacuo*, density was determined in alcohol at 25° and found to be 1.50 for Preparations I and II, 1.48 for Preparation III (see below).

Observations—Two samples of dahlia inulin were used; both were prepared by Dr. R. F. Jackson of the Bureau of Standards, to whom we are greatly indebted. The first (Preparation I), made in large quantity for animal experimentation, was subjected to preliminary purification with milk of lime followed by two recrystallizations from hot water. It was washed with cold water, then with absolute alcohol, air-dried for several days, passed through a 20 mesh sieve, and finally dried at 60° for about 2 hours. This sample contained 9.2 per cent water, 0.05 per cent ash, and 0.01 per cent alcohol. Estimated by the Shaffer-Somogyi method (7), the reducing power of a solution prepared by pouring boiling water on the dry material and cooling after 1½ minutes was equivalent to 0.7 and 0.8 gm. of fructose per 100 gm. of inulin.

Table I presents the results obtained with four different solutions of Preparation I. Each solution was made by heating a mixture of water and inulin to 95° in a water bath. The resulting clear solution was kept at that temperature while the individual determinations were being made on samples at 21.7–23.3°. Inasmuch as the vapor pressure of the solution decreased quite rapidly at 95°, only the first estimation on each solution is listed in Table I. The average of the molecular weight values was 3518, the concurrently determined molecular weight of sucrose (Kahlbaum) averaging 329.

Preparation I was used also for a second series of five estimations. These solutions were made by pouring boiling water on

the dry inulin in a volumetric flask. The inulin dissolved completely within $1\frac{1}{2}$ minutes as a rule. At the end of this period the clear solution was immersed in a water bath at 50° . Table II presents the first molecular weight estimation on each solution.

TABLE I
Molecular Weight of Inulin, Preparation I; Solution Kept at 95°

Solution No.	Inulin per 100 gm. H ₂ O	Reducing power as fructose	Mol. wt. of inulin	Mol. wt. of sucrose*
	<i>gm.</i>	<i>per cent</i>		
1	12.835	0.66	3556	331
2	14.512	0.78	3439	330
3	13.450	0.69	3422	324
4	14.314	0.61	3653	330
Average.....			3518	329
" deviation.....			87	2.3

* Kahlbaum's sucrose was used for the control determinations.

TABLE II
Molecular Weight of Inulin, Preparation I; Solution Made with Boiling Water, Then Kept at 50°

Solution No.	Inulin per 100 gm. H ₂ O	Reducing power as fructose	Mol. wt. of inulin	Mol. wt. of sucrose*
	<i>gm.</i>	<i>per cent</i>		
1	8.864	0.49	4270	338
2	6.450	0.74	4718	
3	6.645	0.86	4850	336
4	8.397	0.61	4291	325
5	10.635	0.93	4156	345
Average.....			4457	336
" deviation.....			262	5.5

* Kahlbaum's sucrose was used except for Solution 5 in which a Bureau of Standards preparation was used.

The average of five determinations was 4457, the concurrently determined molecular weight of sucrose (Kahlbaum) being 336.

When vapor pressure determinations were made consecutively on one solution by withdrawing samples at intervals, the values for molecular weight decreased gradually. As shown in Table III

the apparent molecular weight diminished at 95° from 3422 to 2855 in 140 minutes, and at 50° from 4850 to 4543 in 101 minutes.

It was suspected that the ash and alcohol content of Preparation I might have interfered with the molecular weight estimations. Therefore, a second sample of inulin (Preparation II) was especially purified by Dr. Jackson for further study. After two preliminary crystallizations from water the inulin was washed with alcohol, and then three times recrystallized from water by being dissolved at 85°, filtered through carbon, and allowed to cool to room temperature overnight. The crystals were washed abundantly with distilled water after each crystallization. After the last crystallization the material was spread on a glass plate,

TABLE III

Change in Apparent Molecular Weight of Inulin, Preparation I, on Standing at 95° and at 50°

Inulin per 100 gm. H ₂ O	Time of standing at 95°	Mol. wt. of inulin	Inulin per 100 gm. H ₂ O	Time of standing at 50°	Mol. wt. of inulin
gm.	min.		gm.	min.	
12.835	33	3422	6.45	7	4850
	60	3315		36	4778
	93	3315		79	4794
	127	3080		108	4543
	173	2855			

dried in air first at room temperature then at 55°, and finally pulverized in an agate mortar. According to analysis by the Bureau of Standards the ash content was reduced to 0.008 per cent and the sample contained no alcohol. Further drying to constant weight in a current of dry air at room temperature showed a water content of 10 per cent. A solution prepared by the addition of boiling water, with immediate cooling, had a reducing value equal to 0.5 gm. of fructose per 100 gm. of inulin. Preparation II dissolved in hot water somewhat more slowly than did Preparation I and on cooling began to precipitate more quickly. This prevented satisfactory consecutive estimations on the same solution. •

Table IV presents values for molecular weight obtained with five solutions of Preparation II treated in the same manner as

those of Preparation I used in the experiments summarized in Table II. Since this highly purified inulin did not go into solution so rapidly as Preparation I, it was necessary, on the average, to expose the dissolving inulin to a temperature at or near the boiling point for $2\frac{1}{2}$ minutes. In five determinations the molecular weight of Preparation II averaged 5101, while the concurrently determined molecular weight of sucrose averaged 340.

TABLE IV

Molecular Weight of Inulin, Preparation II; Solution Made with Boiling Water, Then Kept at 50°

Solution No.	Inulin per 100 gm. H ₂ O	Reducing power as fructose	Mol. wt. of inulin	Mol. wt. of sucrose*
	<i>gm.</i>	<i>per cent</i>		
1	10.28 *	0.50	5615	341
2	9.50	0.46	5540	340
3	13.42	0.54	4776	346
4	12.86	0.55	4880	
5	14.66	0.44	4694	334
Average.....			5101	340
" deviation.....			381	3.3

* Preparation supplied by the Bureau of Standards.

TABLE V

Molecular Weight of Inulin, Preparation III, Recovered from Dog Urine

Solution No.	Inulin per 100 gm. H ₂ O	Reducing power as fructose	Mol. wt. of inulin	Mol. wt. of sucrose*
	<i>gm.</i>	<i>per cent</i>		
1	13.63	0.86	3792	334
2	11.88	0.78	3764	347
Average.....			3778	341

* Preparation supplied by the Bureau of Standards.

A third preparation of inulin was made by us from the urine of dogs that had received Preparation I by intravenous injection. The urine was mixed with 6 volumes of alcohol, the precipitate redissolved in water, precipitated with alcohol four times, and recrystallized from water four times. The dry material contained 0.05 per cent ash but was free from creatinine and sodium chloride, both of which had been injected simultaneously with the

inulin. As shown in Table V (two solutions) the average molecular weight of inulin recovered from dog urine was 3778, while the concurrently determined molecular weight of sucrose averaged 341.

DISCUSSION

The integrity of the Hill-Baldes vapor pressure method of determining molecular weight, as used by us in these determinations, is indicated by the sucrose determinations. The average of seven estimations of the molecular weight of Kahlbaum's preparation is 330.6; of seven estimations on a preparation supplied by the Bureau of Standards, 341 (theoretically 342). We are compelled to regard the latter as the purer preparation.

We believe the figures for molecular weight of inulin (Preparation I) recorded in Tables I and II to be too low (1) because a trace of alcohol was present, (2) because the ash content and initial reducing power of the preparation were not minimal, and (3) because the molecular weight values increased from 3518 to 4457 when heating of the solution was reduced.

The molecular weights reported for Preparation II (Table IV) are believed to be more nearly correct because of the greater purity of this material. This conclusion is subject to some reservation because solutions of Preparation II seemed to precipitate more easily. No actual precipitation was observed, however, during the 30 to 45 minutes required for a pair of vapor pressure estimations. The molecular weight values show considerable spread, but, as we have noted above, an error of 3 to 8 per cent may be inherent in the method when used with solutions of very low concentration. Since each determination of molecular weight depends upon the measurement of two vapor pressures (inulin solution and reference solution), there is some chance that the error may be doubled.

We make the necessary assumption that the molecular weight of inulin is not altered by dissolving it in adequate concentration in water with the aid of a minimal amount of heat. Yet the fact that its solubility increases from 4 gm. to 36.5 gm., when the temperature rises from 80 to 100°, indicates that a change of some sort takes place. The vapor pressure studies here reported yield no information concerning the physical nature of that change.

It is conceivable that in reducing the amount of heating (to

avoid hydrolysis) we may have failed to avoid danger in the opposite direction, since solutions tend to precipitate on cooling. Yet the similarity between our results with the purer Preparation II and the results obtained through totally different procedures by Irvine, Haworth, and Berner, give confidence in their collective validity. The agreement between our observations and those of Haworth (elevation of boiling point) deserves special mention, since both techniques were relatively simple, and involved for the first determination of molecular weight approximately the same amount of heating.

Samples of inulin recovered from dog urine yielded molecular weight values much nearer those of Preparation I than those of Preparation II. Since the recovered material resembled the former also with respect to ash content and initial reducing power, the figures are regarded as further evidence that unchanged inulin is excreted in the urine.

SUMMARY

The molecular weights of three samples of dahlia inulin were determined by a thermoelectric vapor pressure technique controlled by concurrent estimations of the molecular weight of sucrose. The molecular weight of the purest available preparation of inulin averaged 5101.

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QUANTITATIVE STUDIES OF THE COMPOSITION OF GLOMERULAR URINE

XIV. THE GLOMERULAR EXCRETION OF INULIN IN FROGS AND NECTURI*

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Preliminary experiments made in 1933 provided basis for the statement that the polysaccharide inulin, after intravenous injection into frogs, is excreted in the glomerular urine in the same concentration as that in the blood plasma (1). This paper contains data of those and of additional similar experiments which support that statement. The information derives importance from the high molecular weight of inulin (approximately 5000 (2)), from evidence that it is not secreted into the urine by the renal tubule, and from the fact that the rate of its plasma clearance in normal dogs is identical or nearly identical with that of injected creatinine (1, 3). From what is now known it appears that the most reliable calculations of rates of glomerular filtration in mammals, including man, are those derived from clearance studies of inulin. It is important, therefore, that evidence of the filtrability of this substance through glomerular membranes be recorded.

EXPERIMENTAL

Methods

The animals used were frogs (*Rana pipiens*, 45 to 75 gm.), decerebrated by crushing the skull and prepared according to Wearn and Richards (4), and *Necturi* (*Necturus maculosus*, 190 to

* A report of these experiments was made before the American Physiological Society, March 26, 1936 (*Am. J. Physiol.*, 116, 160 (1936)). The expenses of the investigation have been defrayed in large part from a grant by the Commonwealth Fund.

250 gm.), anesthetized with urethane and prepared according to the method described by Walker (5). Glomerular urine was collected with the apparatus of Wearn and Richards, care being taken to block the neck or early proximal segment of the tubule and to keep the level of the mercury in the leveling bulb of the apparatus a few mm. above that of the surface of the kidney in order that fluid should not be drawn back from the tubule. Blood was taken from frogs by cardiac puncture into capillary pipettes containing a few grains of dry sodium oxalate; from *Necturi*, by puncture of the descending aorta. Plasma was separated by centrifugation immediately after collection.

Pfanstiehl's c.p. inulin (dahlia) was used in the earlier experiments; a purer sample prepared by Dr. R. F. Jackson of the Bureau of Standards was used in the later ones. It was injected intravenously in dosage which in the majority of the experiments amounted to from 0.15 to 0.32 mg. per gm. About two-thirds of the total dose was given 30 to 45 minutes before the first blood sample was taken, the remainder 15 to 30 minutes later.

The analyses of glomerular urine were made by the Walker-Reisinger ultramicroadaptation of Sumner's method (6, 7), modified to include acid hydrolysis of inulin. In the earliest experiments glucose was determined separately in both plasma and glomerular urine. Since, however, it has been shown conclusively by Walker and Reisinger that the glucose concentrations in plasma and glomerular urine are the same, it is obvious that the separate estimation of glucose and inulin is unnecessary. In the later experiments, therefore, we have determined only the total reducing power of the two fluids after hydrolysis.

Glucose standard solutions were used in preparing the standard color mixtures; consequently our determinations of inulin are somewhat in error because of the greater reducing power of fructose. This is unimportant inasmuch as our chief concern is with comparisons of plasma and glomerular urine, not with absolute values.

The manipulative technique described by Richards, Bordley, and Walker (8) was employed. Details of the procedure, which are not contained in the paper by Walker and Reisinger, are as follows:

1. Into a capillary tube of 0.35 mm. internal diameter introduce

a column of the fluid to be analyzed, 2.5 mm. long, *i.e.* 25 micrometer scale divisions, and 50 micrometer scale divisions of 0.15 N H_2SO_4 . The two columns are separated by a 3 to 5 mm. column of air. Draw the fluid columns well in from the end of the tube, break the tube to convenient length, and seal both ends in the microflame. If several determinations are to be made at once, prepare the other tubes in the same fashion before proceeding to the next step.

2. After thorough mixing of the fluid contents of the capillary tubes by centrifugation place the tubes in a small test-tube containing 1.5 cc. of water and suspend this in boiling water for 15 minutes. Transfer the tubes to the centrifuge and spin them several times to recover condensation water.

3. Open each tube, suck the contents of each into a capillary pipette which contains a little oil at the tip, and draw in another minute amount of oil, so that both surfaces of the fluid column are protected from evaporation.

4. Mount a fresh capillary tube (0.35 mm.) on the microscope stage; charge it successively with 15 scale divisions of the hydrolysis mixture from the capillary pipette, 5 scale divisions of 0.3 N NaOH, and 60 scale divisions of Sumner's dinitrosalicylic acid reagent. The air column between the NaOH and the Sumner's reagent should be at least 1 cm. long. Draw these columns well in from the end of the tube, break it to convenient length, and seal both ends in the flame. Lay it aside until a duplicate tube has been prepared and also all similar tubes which are to be managed in one series of determinations.

5. Prepare to make the standard color mixtures in a series of test-tubes. Measure into each 0.5 cc. of one of the standard glucose solutions. Add 1.5 cc. of Sumner's reagent to each of these tubes, and as nearly as possible at the same time mix the fluids in the capillary tubes by repeated centrifugations. On removal from the centrifuge place these in a test-tube containing 2 cc. of water; place this tube, together with the tubes of glucose standard mixtures, in boiling water for 5 minutes.

6. Color comparisons of the unknowns with the standards are made as described by Walker and Reisinger (6).

A preliminary analysis of plasma was usually made to set the limits of the appropriate series of standard glucose solutions.

Most of the values fell within the glucose range of 30 to 120 mg. per 100 cc.; the intervals of the standard range were 10 mg. per 100 cc. The dilution of the original solution with acid and alkali being 4-fold, this range corresponds to glucose equivalents of inulin from 120 to 480 mg. per 100 cc. When it was expected that the concentration would fall outside this range, the volume of acid used in (1) was altered, the normality being changed to make the acidity of the hydrolysis mixture 0.1 N.

With proper precautions as little as 0.0001 mg. of inulin dissolved in 0.1 c.mm. of fluid can be determined with satisfactory accuracy.

Control Analyses

Table I contains the results of control determinations of the concentrations of solutions of inulin in water and in normal frog plasma. A watery solution of inulin was made which was 10 to 20 times as concentrated as the desired solution for analysis. Its concentration was determined by the macro-Sumner method (7). Various "unknown" dilutions with water or with frog plasma were made of this by a person other than the analyst. Glucose standards were used in the macro- as well as the ultramicrodeterminations, hence the values given in the columns "Found" and "Known" are the glucose equivalents of inulin.

The figures in the first section of Table I show essentially complete agreement between the results of the ultramicroestimations and those calculated from the macrodetermination of the mother solution.

The results of analyses of the frog plasma solutions are on the average 3 per cent too high, 4.8 mg. per cent. This was shown to be due to the presence in the plasma of ultrafiltrable substances which acquire increased reducing power on heating with acid. This statement is based upon the results of experiments which showed (a) that solutions of glucose alone are not changed with respect to reduction of Sumner's reagent by heating with 0.1 N H_2SO_4 for 15 minutes, and (b) that the reducing power of normal frog plasma and of its ultrafiltrate, with or without added glucose, is increased slightly by that treatment. Table II shows the changes which occurred in three samples of plasma and in the ultrafiltrates from them. Dry glucose was added to each sample

of plasma at the outset in order that the final intensities of color should not be too low for accurate comparisons.

These control tests indicate that the method adopted is adequate for deciding the question of the glomerular filtrability of inulin.

TABLE I

Control Determinations of Concentrations of Solutions of Inulin in Water and in Frog Plasma

The volume of solution taken for analysis = 0.24 c.mm.

Solution No.	Concentration			Known	Difference
	Found				
	Duplicates	Average			
Solutions in water					
	mg. per cent	mg. per cent	mg. per cent	mg. per cent	per cent
1	134	136	135	133	+1.5
2	92	96	94	95	-1.0
3	106	112	109	112	-2.7
4	126	130	128	133	-3.7
5	120	124	122	119	+2.5
6	108	112	110	109	+0.9
7	100	102	101	99	+2.0
Mean					-0.07
Solutions in frog plasma					
8	160	166	163	151	+7.9
9	174	182	178	164	+8.5
10	124	128	126	126.5	-0.4
11	116	112	114	112	+1.8
12	308	300	304	312	-2.6
13	288	280	284	292	-2.7
14	208	200	204	193	+5.7
15	260	260	260	254	+2.4
16	180	172	176	166	+6.0
17	268	272	270	261	+3.4
Mean					+3.0

Animal Experiments

Two series of experiments have been made, ten between March 22 and October 27, 1933 and fourteen in February and March, 1935. The results of all but one (omitted because of known

faults in the collection) are included in Table III. In those of the first group reducing power, before and after hydrolysis, was determined both of plasma and glomerular urine, the difference being designated as inulin. In all of these the reducing values, before hydrolysis, were high (equivalent to 142 to 540 mg. per cent of glucose) chiefly because of the partial hydrolysis of inulin which occurs on heating with Sumner's reagent. They were also erratic; in only two was there good correspondence between "glucose" values of plasma and glomerular urine. In six, however, the total reducing power after hydrolysis was approximately the same for plasma and glomerular urine. These are the reasons why attempts to determine glucose separately were abandoned when the experiments were resumed a year and a half later.

TABLE II

Reducing Power As Glucose, Measured in Mg. per 100 Cc., of Plasma Samples and Their Ultrafiltrates

	Experiment I		Experiment II		Experiment III	
	Plasma	Ultrafiltrate	Plasma	Ultrafiltrate	Plasma	Ultrafiltrate
Before hydrolysis.....	394	388	290	304	239	238
After " 	400	398	299	310	253	249
Difference.....	6	10	9	6	14	11

In Table III the arithmetical mean of the differences between the reducing power after hydrolysis of glomerular urine and plasma, the sign of the differences having been taken into account, is -0.18 per cent. When it is considered that the result of every experiment is derived from analyses of three fluids, it seems convincingly clear that the observed differences are the results of analytical error—that the figures as a whole prove identity of concentration of inulin in the glomerular urine and the plasma from which it is separated. The range of plasma inulin concentrations (150 to 1460 mg. per cent) to which this conclusion applies is noteworthy. The three most widely divergent results do not, we think, detract from its validity.

In two experiments with frogs the volume of glomerular urine was sufficient for protein tests; in one it was negative, in the other

TABLE III

Reducing Power, after Hydrolysis, of Glomerular Urine and Blood Plasma from Frogs and Necturi after Intravenous Injections of Inulin

Experiment No.	Date	Glomerular urine		Time of blood sample		Reducing power after hydrolysis				Difference
		Duration of collection	Volume	Blood I	Blood II	Plasma I	Plasma II	Average*	Glomerular urine	

Frogs										
	1933	min.	c.mm.	min.†	min.†	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	per cent
1	Mar. 23	54	‡	15	51	408	440	420	448	+6.7
2	Oct. 4	40	0.2	-12	55	2720	1570	2171	1510	-30.4
3	" 5	42		-5	47	1520	1400	1460	1560	+6.9
4	" 17	46		-7	50	360	352	356	378	+6.2
5	" 18	50		-9	61	496	464	480	496	+3.3
6	" 25	50		-18	66	272	400	336	300	-10.7
7	" 26	49		-21	56	360	296	322	264	-18.0
8	" 26	61		-7	66	296	328	312	324	+3.8
1935										
9	Feb. 13	45	0.35	-8	51	344	170	253	240	-5.1
10	" 20	70	0.4	-3	76	208	178	193	184	-4.7
11	" 22	70	0.15	-2	74	224	204	214	240	+12.1
12	" 26	40	0.10	-5	48	400	292	344	372	+8.1
13	" 28	47	0.3	-5	52	204	211	207	219	+5.8
14	Mar. 1	23	0.23	-6	29	225	200	213	213	0
15	" 4	26	0.1	-5	31	222	182	202	196	-3.0
16	" 6	31	0.17	-6	37	316	258	287	292	+1.7
17	" 6	37	0.2	-8	45	258	248	253	252	-0.4

Necturi										
	1933									
18	June 6	13½		-1½	32½	1410	895	1285	1345	+4.7
1935										
19	Mar. 26	35	0.3	-12	47	172	128	150	144	-4.0
20	" 27	18	0.85	-17	25	276	216	239	232	-2.9
21	" 27	20	0.18	-11	28	216	192	203	210	+3.4
22	" 29	18	0.3	-9	27	338	272	305	316	+3.6
23	" 29	17	0.15	-21	23	272	240	250	272	+8.8

* Interpolated when necessary to the middle of the collection period.

† After or before (-) beginning of glomerular urine collection.

‡ Volume not recorded.

it was recorded as barely positive. Protein tests were also made on three samples of glomerular urine from *Necturi*; one contained a moderate amount, another was recorded as about equal to a 1:75 dilution of plasma, *i.e.* about 0.03 per cent, and the third contained no protein. The conclusion can therefore be drawn that inulin is completely filtrable through glomerular membranes which are impermeable to protein.

In two experiments with frogs in which bladder urine was collected, the urine to plasma concentration ratios of reducing substances after hydrolysis were 15.4 and 8.2; the inulin concentration ratios were doubtless higher than this because of the presence of glucose in the plasma and its probable absence from the urine. The inulin concentration of the first of these urines was calculated to be 22 per cent; this urine solidified on standing.

*Note on Filtrability of Inulin through Artificial Membranes—*Of all of the substances which have thus far been found in glomerular urine in concentrations the same as those in plasma inulin has by far the largest molecule. Its molecular weight is of the order of 5000 and it is believed to consist of a chain of some thirty fructofuranose groups (9). Knowledge that it filters completely through the glomerular membrane therefore contributes something new to a description of this tissue. It was of interest to test its passage through a variety of artificial membranes such as are commonly employed in ultrafiltration and diffusion experiments. Water solutions were used containing inulin in concentrations of from 170 to 500 mg. per cent; they also contained creatinine, 100 to 200 mg. per cent. The membranes used were collodion bags, prepared from du Pont's parlodion according to Greenberg and Gunther (10), du Pont's cellophane Nos. 300, 450, 600, and 1200, and a prepared peritoneal membrane (goldbeater's skin). In summary, the results showed (1) that both substances are completely filtrable through collodion. This is also true when they are dissolved in horse serum. (2) Creatinine is completely filtrable through cellophane No. 300; the concentration of inulin in the filtrates was, however, only from 48 to 93 per cent of that of the original solution (average of five experiments, 74.3 per cent). (3) Creatinine is completely filtrable through cellophane No. 450; inulin concentration of the filtrates varied between 11.6 and 50.0 per cent of the original solution (average of eighteen experi-

ments, 30.4 per cent). (4) With cellophane No. 600, inulin in the filtrate was 2.4 per cent of the original solution; creatinine, 95 per cent (one experiment only). (5) In two experiments with cellophane No. 1200 no inulin was detectable in the filtrate; 35 and 37 per cent of the creatinine. (6) Peritoneal membrane allowed complete passage of creatinine; only 50 per cent of the inulin passed through.

Notes on Diffusibility of Inulin—A few experiments have been made to test the supposition that inulin is more slowly diffusible than are the constituents of normal urine. A typical experiment is as follows: 100 cc. of a watery solution containing inulin, 8.35 per cent, creatinine, 0.235 per cent, and NaCl, 0.217 per cent, were placed in a collodion bag and this suspended in 100 cc. of water at room temperature (25°). The two fluids were stirred

TABLE IV
Percentage Diffusion of Constituents of Watery Solution after Varying Periods

	30 min.	73 min.	143 min.	317 min.
Inulin.....	6.7	14.7	25.0	42
Creatinine.....	19.4	34.6	44.0	49.5
NaCl.....	26.8	42.5	50.0	51.5

frequently and equal samples of both fluids taken at intervals for analysis. The results are given in Table IV.

In six experiments the free diffusion of these three substances was tested. The apparatus used was kindly loaned by Dr. Samuel Goldschmidt and had been used by him in his studies of the movement of ions (11). It consists essentially of a large stop-cock tube of such dimensions that the capacity of a well in the stop-cock is slightly more than 11 cc. By turning the stop-cock the well may be made to communicate with either end of the tube. The apparatus, the solution, and the solvent (water) were warmed to incubator temperature, the stop-cock well was filled from one end of the tube with the solution, the stop-cock was turned through 90°, and that end of the tube was carefully washed and dried. A volume of pure water, equal to that of the solution in the stop-cock well, was measured into the other end of the tube, and subse-

quent evaporation was prevented by a glass stopper. The apparatus was then placed upright in the incubator (35°, regulated to 0.05°). After waiting about 3 hours for the temperature of the system to become constant, the stop-cock was carefully turned, again through 90°, so that the two fluids were in contact (solution below, solvent above). After about 20 hours the stop-cock was carefully turned and the two fluids, again separated, were drawn off separately for analysis. Six experiments were made; the results of one, expressed as percentage of solute which diffused into the water in 20½ hours, are as follows.¹ They are typical of the group.

Inulin.....	6.5
Creatinine.....	18.1
NaCl.....	23.2

DISCUSSION

The information obtained in these experiments adds an important item to existing evidence that the glomerular process in Amphibia consists solely of filtration. Every substance thus far studied quantitatively (NaCl, P₂O₅, NaHCO₃ + CO₂(pH), glucose, urea, uric acid, creatinine, phenol red, indigo carmine, and now inulin) has been found in the glomerular urine in the same concentration as that in which it exists free in the plasma. Among them are substances indispensable to the body and reabsorbed from the tubule; others must be excreted if the animal is to survive; still others are substances foreign to the body. All pass through the glomerular membrane alike. This fact is irreconcilable with a view that active processes operate within the glomerular membrane to accelerate or retard the passage of individual solutes through it.

It is also incredible that solution in the substance of the membrane can play a part in the passage of such a variety of substances through the membrane at equal rates with respect to concentrations in the plasma. None has a significant degree of lipid solubility; their water solubilities differ widely.

That diffusion or osmosis is not a significant factor is indicated

¹ The solution contained 0.5 gm. of inulin, 0.2 gm. of creatinine, and 1.0 gm. of NaCl per 100 cc.

by the observations on the diffusibility of inulin in comparison with that of creatinine or NaCl. If it were, the concentration of inulin in the glomerular urine would be lower than that in plasma.

Our results, we think, furnish safe ground for belief that the passage of plasma water and its contained solutes through the glomerular membrane takes place through physical openings or pores of such dimensions that the molecule of inulin, presumably hydrated (12), can pass through; and it becomes incredible that the membrane could retard the passage of the far smaller molecules of the normal urinary constituents.

Ekehorn in 1931 propounded a theory which he designated as "the 'protein-proof' of the nature of the process of glomerular exudation" (13). Its essential points, as we understand them, are these: A kidney, the glomeruli of which, because of injury, permit leakage of plasma protein, is not *necessarily* abnormal in any degree with respect to its capacity to excrete the normal constituents of urine. The passage of protein through such injured membranes can be due to nothing else than filtration, as through the meshes of a sieve; and molecules of normal urinary constituents, vastly smaller than those of protein, must certainly be capable of passing through the same openings as a result of the same force (pressure). If the kidney, whose glomeruli leak protein, excretes these smaller molecules in wholly normal fashion, it follows that there can be no difference in their mode of passage through the injured and the normal glomerular membranes. The glomerular process in normal glomeruli therefore is filtration.

This argument appears to be based upon the assumption that the entire glomerular surface of the injured kidney is so changed that everywhere a trace of protein leaks through. It is, however, equally permissible to assume that here and there in the glomerular surface there is a gross defect which allows the escape of a little whole plasma, the rest of the membrane being normal. 30 gm. in 24 hours is a large amount of protein to be excreted by a human patient whose kidney function is otherwise normal. If the concentration of plasma proteins in such a patient is 4.0 per cent, the protein leakage is the equivalent of 750 cc. of plasma. A conservative estimate of his glomerular elimination is 144 liters in 24 hours, *i.e.* about 200 times the plasma leakage. If the alternative assumption suggested above is accepted as a possibility, it is

obvious that Ekehorn's arguments may be inapplicable to some 99.5 per cent of the glomerular surface.

In the experiments which we have described we find that a substance whose molecule is some 25 times as large as that of any normal constituent of urine passes through the normal, protein-tight, glomerular membrane with the same speed as do the latter. We think that this fact gives the basis for a first approximation to the dimensions of the passages through the normal, glomerular membrane which is more credible than can be one derived from considerations of protein leakage.

SUMMARY

An ultramicromethod for the quantitative determination of inulin is described. In control experiments its accuracy was shown to be adequate for the quantitative comparison of plasma and glomerular urine with respect to inulin concentration.

Results of twenty-three experiments with frogs and *Necturi* are detailed which show that intravenously injected inulin is excreted in the glomerular urine of these animals in concentrations which are the same as those found in the plasma.

Inulin was found to be completely filtrable through collodion membranes prepared according to Greenberg and Gunther and incompletely filtrable through cellophane Nos. 300, 450, and 600, and through a commercial peritoneal membrane. Creatinine was completely filtrable through all of these. Protein was filtrable through none.

Free diffusion experiments showed the mobility of inulin molecules to be significantly less than those of creatinine and of the ions of NaCl. Its diffusion through artificial membranes is slower. These observations together with the identity of inulin concentration in glomerular urine and plasma indicate that diffusion plays no significant rôle in glomerular function.

Since the molecular weight of inulin is of the order of 5000 or more and the dimensions of its molecule are far larger than those of any of the normal urinary constituents, the results summarized are believed to constitute important additional evidence that the glomerular process is one of filtration solely and to provide the basis for a first approximation to the dimen-

sions of the pores in the glomerular membrane through which the glomerular filtrate is expressed.

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INULIN AND CREATININE CLEARANCES IN DOGS, WITH NOTES ON SOME LATE EFFECTS OF URANIUM POISONING*

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The interest which attaches to the study of the rate of renal excretion of inulin in mammals derives from the large molecular dimensions of that substance and from the evidence which permits belief that it is excreted solely by glomerular filtration. The rate of its plasma clearance can be accepted as the equivalent of the rate of glomerular filtration with fewer reservations than are necessary in the case of other substances which have been similarly studied, such as creatinine, xylose, or glucose in phlorhizin poisoning. And the most important outcome of the measurements of the magnitude of its plasma clearance is the evidence which they afford that the volume of the glomerular filtrate in dogs and rabbits is great enough to contain all of the normal constituents of urine except such as are formed within the kidney itself.

Independent work from three laboratories has established the approximate identity of the plasma clearances of injected inulin and creatinine in dogs, thus validating Rehberg's hypothesis (1) as applied to dogs. Our published figures (2) of inulin to creatinine clearance ratios gave a mean of 1.06 with a standard deviation of 0.20; those of Shannon (3) gave a mean of 1.006, standard deviation, 0.034; those of Van Slyke, Hiller, and Miller (4), 0.97, standard deviation, 0.14. While the general conclusion from each of these groups of measurements is the same, the remarkable consistency with which Shannon's ratios approximate to unity

* The expenses of this work have been defrayed in large part from a grant by the Commonwealth Fund.

gives a special significance to his results. They mean not only that the two substances are excreted at exactly the same rate relative to their concentrations in plasma, but also that neither substance is reabsorbed from the tubule, either actively or by diffusion. Our ratios could mean that variable, usually small, fractions of the creatinine of the glomerular filtrate are reabsorbed from the tubule. We became convinced that the technique of Shannon's experiments was more perfect than ours and hence were inclined to accept the more rigid conclusion drawn from his results. But at the same time, in view of the great difference between the diffusibilities of inulin and creatinine (5), we found it difficult to believe that the normal dog tubule is possessed of such constant, specific impermeability as never to permit the escape by diffusion of any of the creatinine from the fluid within its lumen. We have therefore continued to collect data concerning clearance of these two substances in dogs, first however having somewhat modified our analytical methods in accordance with suggestions from Professor Smith and Dr. Shannon in order to lessen chances of addition of errors.¹ In the meantime Shannon has published a second group of observations on dogs the results of which confirm his earlier work and extend its conclusion to include low rates of urine flow (6).

In our experiments ten normal and two abnormal dogs were studied. These two (Dogs SH and RD), loaned by Dr. MacNider, had been poisoned with uranium more than 2 years before, but now look perfectly well.² The results obtained in all except one of the abnormal dogs coincide so closely with those obtained by Shannon as to uphold the conclusion drawn from his work. In the experiments on one of the abnormal dogs, however, we have found the inulin to creatinine clearance ratio to be so consistently divergent from 1.00 as to indicate the reabsorption of some of the creatinine from the glomerular filtrate.

¹ Dr. Shannon very generously came to Philadelphia and collaborated with us in one of the experiments.

² Dog SH received 2 mg. of uranium nitrate per kilo by subcutaneous injection on June 3, 1932 and again on February 17, 1933. Our first experiment with Dog SH was on January 24, 1936. Dog RD was injected with 2 mg. per kilo on June 1, 1933. Our first experiment was made on January 30, 1936.

Methods

Female dogs, weighing 13.0 to 19.4 kilos, accustomed to the experimental routine of catheterization and venipuncture, were used. During intervals between experiments they were kept on the ordinary mixed diet of the animal house and were given no food for at least 20 hours before an experiment. In all experiments save one, water was given by stomach tube in varying dosage and at varying times before the beginning of the experiment. The largest amount was 140 cc. per kilo in divided amounts during 6 hours before; the smallest, 15 cc. per kilo 1 hour before. Inulin was given by intravenous injection (saphenous vein) in all experiments except one in which injection was subcutaneous. Creatinine was given by subcutaneous injection in seven experiments, intravenously in seventeen. Pfanstiehl's c.p. dahlia inulin was used in twelve experiments; an especially purified preparation from the Bureau of Standards was used in 12. Pfanstiehl's creatinine was used in all.

Urine was collected by catheter; urine periods in different experiments varied from 10 to 30 minutes in length. Complete emptying of the bladder was accomplished by abdominal compression after inflation of the bladder with air, or injection of warm salt solution, or both. Blood was taken from the jugular vein at the midpoint of each urine period and centrifuged immediately. In the majority of experiments dry lithium oxalate was used as anticoagulant; in a few, heparin. Analytical methods for inulin and creatinine were the same as those used by Shannon (3, 7) with the exception that glucose was determined in the iron filtrates (8) by the Shaffer-Somogyi method (9) instead of being removed by fermentation with yeast. Urea in experiments with Dogs SH and RD was determined by the method of Van Slyke and Cullen (10). All determinations were made in duplicate.

Results

The clearance measurements were made within a wide range of experimental conditions, as represented by the following summary. Rates of urine flow varied between 0.32 and 10.0 cc. per minute, plasma inulin concentrations between 16 and 1292 mg. per cent, plasma creatinine between 8 and 112 mg. per cent, urinary inulin

between 0.33 and 29.3 per cent, urinary creatinine between 0.16 and 3.74 per cent.

The inulin and creatinine clearances and their ratios are charted

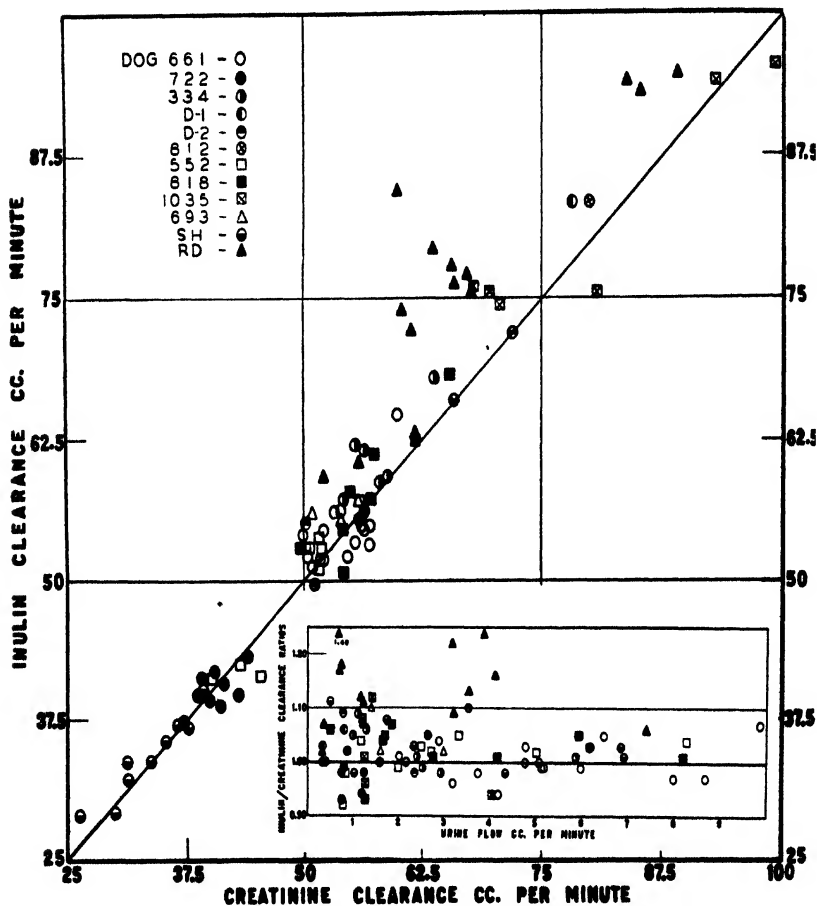


FIG. 1. Inulin clearances plotted against creatinine clearances for each period in each dog. The inset shows inulin to creatinine clearance ratios plotted against rates of urine flow.

in Fig. 1. Exclusive of the results obtained with Dog RD, the mean of 73 inulin to creatinine clearance ratios derived from eighteen experiments on eleven dogs is 1.016, the maximum variations $+0.103$ and -0.097 , the standard deviation 0.044 , the

standard error of the mean 0.005. These figures so closely resemble those of Shannon as to force us to accept his conclusion that in normal dogs neither inulin nor creatinine is reabsorbed from the tubule in significant amounts.

The results obtained with the uranium-poisoned animal, Dog RD, however, are consistently divergent from the rest. The lowest of fourteen inulin to creatinine clearance ratios is 1.02, the mean 1.152, the standard deviation 0.109, the standard error of the mean 0.030. The only meaning which we can attach to this difference is that in this dog, an average of at least 13 per cent of the creatinine contained in the glomerular filtrate is reabsorbed from the tubule. Nothing which we know of the sequelæ of uranium poisoning permits belief that active tubular processes are increased by it; hence we ascribe the difference between Dog RD and the others to impairment of the normal impermeability of the tubule in the direction lumen \rightarrow blood with the result that some escape of creatinine occurs by diffusion. It is obvious from the high inulin clearances that little, if any, escape of inulin occurred.

Other comparisons between Dogs RD and SH (the latter had also been poisoned with uranium) support the above interpretation.

Examination at Biopsy—The kidneys of Dog SH were described as tough and fibrous, those of Dog RD as friable, "sutures cut through easily."

Microscopic. Dog SH—Sections of kidney tissue taken at eight biopsies made at intervals between the first uranium poisoning (June 8, 1932) and the beginning of our experiments (January 24, 1936) show marked progressive glomerular change which has resulted in fibrosis and atrophy (including complete disappearance) of many glomeruli and tubules. Compensatory enlargement of many of the existing nephrons is very evident.

Dog RD—Sections of kidney tissue taken at biopsy 4 months after the uranium poisoning show normal glomeruli, vacuolization of cells of the convoluted tubules, nuclei of tubule cells, normal. Tissue taken 8 months and 1 month before the beginning of our experiments show practically normal kidney, no atrophy of nephrons, or fibrosis.³

³ Dr. Lucké informs us that failure to find visible evidence of abnormalities of the tubule cells is not necessarily a reason for doubting the interpretation which we have given to the clearance results.

Inulin Clearances—Those of Dog SH were the lowest of any of the twelve dogs studied. The highest of nine measurements was 40.3; the lowest, 29.0 cc. per minute; average, per sq. m. of body surface, 43.3. The inulin clearances of Dog RD were among the highest; the lowest of fourteen was 78.2; the highest, 126.0; average per sq. m., 104.0 cc. per minute.

Urea Clearances—These were measured simultaneously with those of inulin and creatinine. For Dog SH the mean of nine urea to inulin clearance ratios is 0.60 ± 0.033 (the standard error of the mean); for Dog RD the mean of fourteen ratios is 0.347 ± 0.020 .

When comparison is limited to periods in which rates of urine flow were approximately the same in both dogs, the mean of seven urea to inulin ratios for Dog SH is 0.56; of seven for Dog RD, 0.35.

Other Tests

	Dog SH	Dog RD
Maximum sp. gr. after 48 hrs. deprivation of water*.....	1.042	1.041
Phenolsulfonephthalein excretion after 6.0 mg. intravenously (2 tests)		
1st hr., %.....	34, 45	63, 82
2nd " %.....	44, 59	73, 92
Urinary protein in 24 hrs., gm.....	1.5	Trace

* Two normal dogs similarly tested gave urines with specific gravities of 1.050 and 1.052.

From these data we conclude that the kidneys of Dog SH have suffered considerable glomerular damage as a result of which the volume of glomerular filtrate is abnormally low. The capacity of the tubules to retain creatinine and urea is normal; the fraction of filtered urea reabsorbed is 40 per cent. Dog RD, on the other hand, survived the poisoning with no residuum of glomerular damage, detectable either microscopically or by inulin clearance measurements. The capacity of the tubules, however, to retain creatinine is somewhat less than normal; the high fraction (65 per cent) of filtered urea which is reabsorbed gives further evidence of abnormal permeability of the tubule.

The inability of Dog SH to excrete a urine as concentrated as that of normal dogs is to be ascribed to decrease in filtration surface. The similar inability shown by Dog RD is due to diminished impermeability of the tubules.

Grateful acknowledgment is made to Dr. Wm. deB. MacNider for the uranium-poisoned dogs and the sections of kidney tissue from them, to Dr. Balduin Lucké for his histological study, and to Miss Ethel Shiels for the urea determinations.

SUMMARY

Plasma clearances of injected inulin and creatinine have been measured in experiments in twelve dogs, of which ten were normal and two had been poisoned with uranium more than 2 years before these tests. In eleven of these dogs, within wide ranges of rates of urine flow and plasma and urine concentrations, the clearances were found to be so nearly the same as to support the view that in normal dogs, under all ordinary experimental circumstances, these two substances are excreted at the same rate with respect to their concentrations in plasma. The observations add to the volume of existing inferential evidence, not only that the dog kidney excretes both substances solely by glomerular filtration, but also that neither substance is reabsorbed from the tubule, either actively or by diffusion.

In one of the poisoned dogs inulin was consistently excreted at a faster rate than creatinine. This fact is interpreted to mean that the impermeability of the tubule which normally prevents back diffusion was so impaired that a fraction of filtered creatinine, amounting on the average to at least 13 per cent, returned to the blood. The renal damage established a condition in which the greater diffusibility of creatinine was revealed.

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PROVITAMIN D POTENCY OF SOME STEROL DERIVATIVES*

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In a previous paper (1) we reported that spinal cord cholesterol which has been purified through the dibromide, thus removing all trace of the contaminant responsible for the four absorption bands associated with the presence of ergosterol, is, when heated above its melting point and irradiated, equivalent in terms of rat units to cod liver oil and to the original spinal cord cholesterol in preventing avian rickets. These results have since been confirmed by Hathaway and Lobb (2) and by Haman and Steenbock (3). The latter authors publish an absorption spectrum for heated, purified cholesterol which shows absorption bands similar to that of their original crude cholesterol but shifted somewhat to the red end of the spectrum. We have submitted several samples of heated cholesterol to Professor T. R. Hogness and Dr. Fred P. Zscheile for spectrographic analysis. They have never observed peaks at 2600, 2700, 2820, and 2935 Å., but instead have found a broad band between 2250 and 2600 Å., the height of which appears to parallel roughly the provitamin D potency. While the evidence associating this band with antirachitic action is not yet conclusive, we are at least convinced that the provitamin D of our heated purified cholesterol is quite different from the provitamin D of spinal cord cholesterol which does exhibit the four absorption bands earlier associated by Windaus and Hess (4) and Rosenheim and Webster (5) with the presence of ergosterol.

Waddell's findings (6), which we (1) later confirmed, that spinal cord cholesterol was more effective in preventing leg weakness

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than either ergosterol or purified cholesterol plus ergosterol, indicated that the contaminant of spinal cord cholesterol is not ergosterol but some derivative of cholesterol which also has the two double bonds in Ring B and which therefore also shows the four typical absorption bands.

EXPERIMENTAL

When Windaus, Lettré, and Schenck (7) described the preparation of 7-dehydrocholesterol and reported it about one-half as

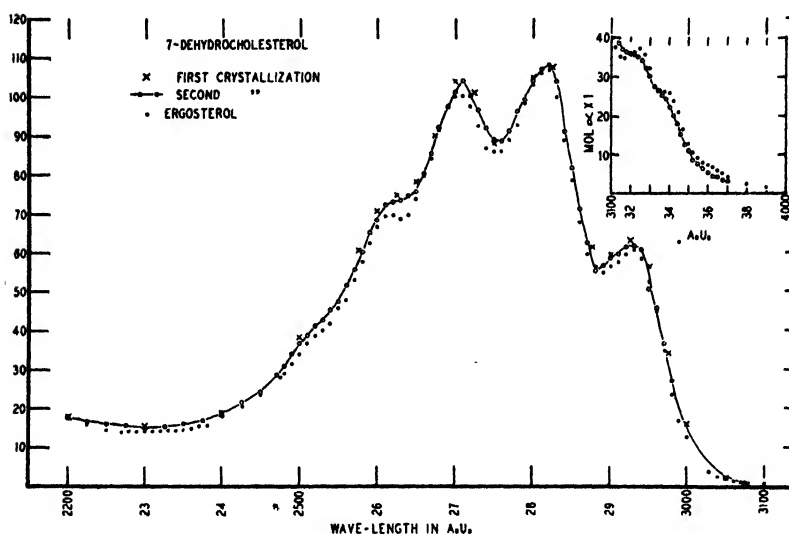


FIG. 1. Absorption spectra of 7-dehydrocholesterol and ergosterol in ethyl alcohol. This figure was prepared by Professor Hogness and his group.

potent for rats as irradiated ergosterol, we immediately started to make some of this product, with the idea of determining its effectiveness for chicks. We obtained about 1 gm. of pure white crystals which, when irradiated *dry*, were only one-fifteenth to one-tenth as potent for rats as ergosterol activated in the same way. Two successive recrystallizations of this product from alcohol and two recrystallizations of ergosterol from alcohol were submitted to Dr. Hogness and Dr. Zscheile. They obtained four curves which were superimposable on the molecular basis, the

TABLE I

Chick Assay. Comparative Antirachitic Potency of Cod Liver Oil, Viosterol, Irradiated Corn Oil Phytosterol, and Irradiated 7-Dehydrocholesterol

Supplement to basal ration per 100 gm.	Rat units per 100 gm. diet	Chick assay				Remarks
		Average weight at 28 days	Average gain in weight	Line test	Bone ash	
		gm.	gm.		per cent	
None	0	123	75	Rickets	29.0	All symptoms of leg weakness; tendency to squat at 10 days
0.250 gm. cod liver oil	10	189	144	1 mild rickets, 8 good calcification	41.5	2 to 3 showed tendency to squat in 4th wk.
0.05 gm. viosterol (old sample*) diluted 100 times	100	177	132	6 rickets, 1 mild rickets, 3 good calcification	38.0	" "
0.15 gm. viosterol (old sample*) diluted 100 times	300	177	131	4 slight rickets, 7 good calcification	44.0	Chicks active, feathers rough
15 mg. irradiated corn oil phytosterol	35	173	127	8 mild rickets, 3 good calcification	37.1	Chicks active, 1 to 2 showed tendency to squat 4th wk.
0.2 mg. irradiated (dry) 7-dehydrocholesterol	10	181	135	Good calcification	45.1	Chicks strong and active, feathers smooth
1.0 mg. irradiated 7-dehydrocholesterol	50	191	145	" "	44.9	" "
2.0 mg. irradiated 7-dehydrocholesterol	100	185	136	" "	46.0	" "

* 5000 times the strength of cod liver oil.

formula of 7-dehydrocholesterol being assumed to be $C_{27}H_{44}O$ (see Fig. 1). This was apparently, then, a product of high purity, and in spite of the low potency of the dry irradiated crystals, we compared their effectiveness in preventing leg weakness with that of cod liver oil, viosterol, and dry irradiated corn oil phytosterol.

By all the criteria (see Table I), weight gain, line test, bone ash, and appearance of the chicks, the group receiving 10 rat units of irradiated 7-dehydrocholesterol per 100 gm. of the Lachat, Halvorsen, and Palmer (8) diet were more completely protected against leg weakness than those receiving 0.25 per cent or 10 rat units of cod liver oil or 300 rat units of viosterol in 100 gm. of diet. The bone ash, 45.1 per cent for Group VI as compared with 41.5 per cent for Group II, indicates that this dosage of 7-dehydrocholesterol is considerably more than equivalent to 10 rat units of cod liver oil. The results for Group V receiving irradiated phytosterol are of interest in view of Windaus' (7) finding that 22-dihydroergosterol is one-thirtieth as active for rats as ergosterol, and McDonald's (9) report that, rat unit for rat unit, its efficiency for chicks is intermediate between cod liver oil and ergosterol. In our experiment, 35 rat units of irradiated corn oil phytosterol were fully equivalent for chicks to 100 rat units of viosterol. This difference in response of rats and chicks indicates that something other than ergosterol, perhaps 22-dihydroergosterol or possibly a C_{29} derivative of phytosterol, or stigmasterol, is responsible for the four bands exhibited in the absorption spectrum of this phytosterol preparation.

Despite the fact that the absorption spectra indicated that our 7-dehydrocholesterol preparation was a pure product, we found that the melting point was not a definite one, that is 142–143.5°, as reported by Windaus (7). The first lot of crystals from ether-methyl alcohol, after being dried in a desiccator overnight, melted at 135–137°. Recrystallized from ethyl alcohol, they melted at 126°. After thorough drying under a vacuum pump in preparation for spectroscopic analysis, this product melted at 122°. A second crop of crystals from ether-methyl alcohol melted at 119–120°, and after several weeks in a desiccator at refrigerator temperature at 115°. This last product freshly recrystallized from 95 per cent ethyl alcohol melted at 124–126°; recrystallized from ether-methyl alcohol, the air-dried crystals softened at 112° and

melted completely at 120°. After 2 days in a vacuum desiccator at refrigerator temperature, the crystals began to shrink at 126° and with increasing temperature they gradually softened like melting snow until at 140° they formed a transparent globule. In fact the melting points of various crops of crystals from the two solvents, 95 per cent alcohol and ether-methyl alcohol, covered a range of 115–140°; only one sample melted at as high as 140°, two melted at 135–138°. Recrystallization from alcohol never produced a melting point higher than 126°. All melting points were taken in sealed tubes. This shifting melting point indicates that we were working with a mixture of substances, or that 7-dehydrocholesterol changes rapidly to another form, or that, like ergosterol, it easily forms alcoholates and hydrates. Bills and Honeywell (10) report the melting point of ergosterol to vary between 166–183° depending upon the water content of the sample.

From the ether-methyl alcohol mother liquor of the first recrystallization of 7-dehydrocholesterol, a second product separated which melted at 92–95°. Its absorption spectrum showed only a broad band between 2400 and 2800 Å. with no suggestion of four distinct peaks. Nevertheless, when irradiated dry, it was about one-third as potent as the first product. The quantity obtained was insufficient to test on chicks.

To obtain more of these products, we accumulated samples which had been set aside for reference at every step in the preparation of the 7-dehydrocholesterol. These were carried through the necessary final steps. The reactions involved in making 7-dehydrocholesterol are: oxidation of cholesterol acetate with CrO_3 in acetic acid at 55°, reduction of the 7-ketocholesterol acetate thus formed with aluminum isopropylate, hydrolysis of the acetate, and treatment of the 3,7-hydroxycholesterol with benzoyl chloride in pyridine to form the dibenzoate. Small portions, 0.5 gm., are heated for 1½ hours at 200° under reduced pressure. At this stage, 1 molecule of benzoic acid splits off at the 7-8 carbons, introducing a double bond at this point. The monobenzoate remaining is saponified to produce 7-dehydrocholesterol.

In our second preparation we used a variety of solvents to recrystallize the dibenzoate. From acetone, two fractions sepa-

rated, one melting at 151° and one at 181°. Windaus reported the melting point of the dibenzoate to be 171.5–172°. When the two dibenzoates melting at 151° and 181° were heated to split off benzoic acid, their behavior was very different. The first product began to decompose at 190°, and the residue left after 1½ hours at this temperature hardened to a brown glass, similar to what we had obtained in our first preparation. The dibenzoate with the higher melting point did not begin to split off benzoic acid below 200–205°, and the evolution was more rapid at 215–220°. The residue left in this case was a creamy semicrystalline mass. These residues were dissolved in a small volume of chloroform and acetone was added to precipitate the monobenzoate. In our first preparation the yield from 0.5 gm. of dibenzoate averaged 0.13 gm., about 33 per cent of the theoretical; in the second preparation the yield from the lower melting dibenzoate was 0.1 gm. and from the higher melting product, 0.35 gm. or 85 per cent of the theoretical. Windaus reports his yield as 58 per cent of the theoretical. The melting point of the first monobenzoate was 137°, of the second 175°. Windaus describes this product as becoming turbid at 139–140° and clear at 183°. Our original monobenzoate softened at 137° and melted between 163–171°. It appears that our first monobenzoate and Windaus' comprised a mixture of two or more substances.

In our first preparation we obtained by hydrolysis of the monobenzoate, two products, one showing an absorption spectrum identical with that of ergosterol and a melting point ranging from 115–140° and another having a broad band between 2400 and 2800 Å. and a melting point of 92–95°. The low melting monobenzoate of the second preparation yielded only the product melting at 92–93°. Hydrolysis of the higher melting monobenzoate resulted in two ether-soluble compounds. One of these was precipitated with petroleum ether and melted at 171°. The other ether-soluble compound had a melting point of 141.5–142.0°, close to that reported by Windaus. Recrystallization from petroleum ether-methyl alcohol, ethyl ether-methyl alcohol, and from 95 per cent alcohol did not change this melting point.

Samples of our various products having melting points of 125°, 115°, 92°, and 141.5° were irradiated 1 hour in ether solution and assayed on rats. The specific rotation in chloroform solution was

determined. Micropolariscope tubes of 1 dm. length were used, one holding 1.5 cc. and the other 0.2 cc. Duplicate determinations with the two tubes checked closely. Mr. Durey Peterson was good enough to run the microanalyses of these products. The results are given in Table II.

Obviously irradiation in ether solution has given a much more potent product than irradiation of the dry crystals. The first two samples, of melting points 125° and 115° , have a potency approaching that reported by the Windaus group, 0.2 microgram

TABLE II
Properties of Products Obtained in Preparation of 7-Dehydrocholesterol

Solvent used for crystallisation	Melting point	Specific rotation α_D^{25} in chloroform	Mg. \propto 1 rat unit	Analysis and suggested formulae
	$^{\circ}\text{C.}$	<i>degrees</i>		
95% alcohol	Fresh			
	124-126			
	Dry	-107.02	0.0002	C 79.4, H 11.7
	117-119.5			$\text{C}_{27}\text{H}_{44}\text{O}_2 \cdot \frac{1}{2}\text{C}_2\text{H}_5\text{OH}$
Ether-methyl alcohol	115-116	-98.88 to -98.97	0.0002	Recrystallized, m.p. 135-140 $^{\circ}$
				C 81.50, H 10.85
" "	92-93	-24.24	0.001	$\text{C}_{27}\text{H}_{44}\text{O}_2$ or $\text{C}_{27}\text{H}_{42}\text{O}_2$
				C 77.4, H 10.88
Alcohol	141.5	-50.45 to -49.69	0.0005	$\text{C}_{27}\text{H}_{44}\text{O}_2$ or $\text{C}_{27}\text{H}_{44}\text{O}_2$
				C 83.8, H 11.8
Ether-petroleum ether	171.0		No potency	$\text{C}_{27}\text{H}_{44}\text{O}$
				C 79.4, H 11.4
				$\text{C}_{27}\text{H}_{44}\text{O}_2$

per rat unit as compared with 0.15 microgram. The product of higher melting point was one-third to one-half as potent, the product of low melting point is only one-fifth as potent.

No sample had as high a levorotation as that reported by Windaus *et al.*, *i.e.* $\alpha = -113.6^{\circ}$. The microanalyses for the two products of highest potency which have an absorption spectrum like that of ergosterol do not fit the formula $\text{C}_{27}\text{H}_{44}\text{O}$ for 7-dehydrocholesterol. They point to the presence of an aldehyde, a hydroxy or possibly a ketone group, either on a ring carbon or in the chain giving a composition of $\text{C}_{27}\text{H}_{44}\text{O}_2$ or $\text{C}_{27}\text{H}_{42}\text{O}_2$ or $\text{C}_{27}\text{H}_{40}\text{O}_2$.

The analysis of the recrystallized sample from alcohol fits best the formula $C_{27}H_{44}O_2 \cdot \frac{1}{2}C_2H_5OH$, indicating that an alcoholate is formed. Crystals from alcohol appear much more stable than those from ether-methyl alcohol. Kept in a desiccator at refrigerator temperature, they remained white and definitely crystalline for at least 6 months, while the crystals from ether-methyl alcohol turned somewhat yellow and matted together. The analysis of the low melting product corresponds to a compound with two additional hydroxyl groups or possibly two ketones. On standing it tends to become gummy and is not easily recrystallized from any solvent thus far tried. Differentiation between cholesterol and 7-dehydrocholesterol can scarcely be determined by this analytical technique. The analysis of the product melting at 141.5 – 142° is of little significance in determining the possible contamination with cholesterol.

Solutions of 2 mg. per 100 cc. in redistilled ethyl alcohol were compared in a Bausch and Lomb spectrograph with a solution of 1 mg. of ergosterol per 100 cc. We have determined that the four bands of ergosterol can just be detected in a solution of 0.0001 mg. per 100 cc. in a 100 mm. cell with 2 seconds exposure. By dilution or by varying the length of the cell, we can arrive fairly quantitatively at the concentration of ergosterol in solution. Examination of Fig. 2 shows that the first two samples melting at 126° and 115° respectively have an absorption intensity approximately equal to that of ergosterol; *i.e.*, 2 mg. per 100 cc. in a 50 mm. cell (see *C* and *E*, *K* and *M*, Fig. 2) give a picture similar to that produced by 1 mg. per 100 cc. of ergosterol in a 100 mm. cell (see *N*). The second product, melting at 92 – 93° , shows no distinct bands, but absorbs weakly at 2600 to 2400 Å. (see *F* and *G*). The last product, melting at 141.5° , shows the four bands, but the absorption is about one-fourth the intensity of ergosterol; *i.e.*, 2 mg. per 100 cc. in a 100 mm. cell (see *H*), is about equivalent to 1 mg. of ergosterol per 100 cc. in a 50 mm. cell (see *O*).

The lower antirachitic potency, the specific rotation, the analysis, and the absorption spectrum of the product melting at 141.5 – 142° all suggest that it may consist of 7-dehydrocholesterol contaminated with unchanged cholesterol, or rather of cholesterol mixed with one-fifth to one-fourth the amount of 7-dehydrocholesterol. On the other hand, the constancy of melting point and

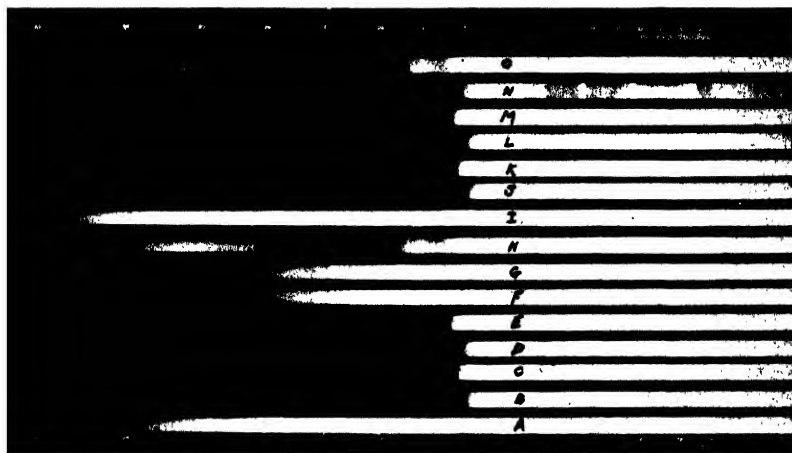


FIG. 2. Absorption spectra of the various products with an exposure of 2 seconds. Unfortunately the quantitative differences do not show as distinctly in this reproduction as in the negative. These observations have been confirmed by Professor Hogness and his group.

		Concentration	Length of tube
		mg. per 100 cc.	mm.
A	Ethyl alcohol-solvent		100
B	Product I, m.p. 126°, crystallized from alcohol	2	100
C	Same	2	50
D	Product I, m.p. 115°, crystallized from ether-methyl alcohol	2	100
E	Same	2	50
F	Product II, m.p. 92-93°, crystallized from ether-methyl alcohol	2	100
G	Same	2	50
H	Product III, m.p. 141.5-142°, crystallized from alcohol	2	100
I	Same	2	50
J	Product I, m.p. 135-138°, crystallized from ether-methyl alcohol	2	100
K	Same	2	50
L	Product I, m.p. 115°, crystallized from ethyl-methyl alcohol	2	100
M	Same	2	50
N	Ergosterol	1	100
O	"	1	50

specific rotation with recrystallization points to a single compound. This is the only product we have obtained with a melting point comparable to that reported by Windaus. Also the melting point of crude cholesterol benzoate which we prepared is 149.5° , while the dibenzoate from which this product was made showed no indication of softening below 180° . We have under way preparation of larger quantities of these products. Of especial interest to us is the substance melting at $92-93^{\circ}$, since its absorption spectrum is similar to that of our heated cholesterol preparation. It is an exceedingly unstable substance changing to a gum in a manner similar to the bile acids, and gradually losing its provitamin potency. Obviously, in the preparation of 7-dehydrocholesterol many side reactions occur. Further study of these products and their reactions is necessary to determine their identity.

Antirachitic Potency of Other Sterol Derivatives—Parallel with these studies we have been testing various other sterol derivatives for possible antirachitic action. The intermediate products in preparation of 7-dehydrocholesterol (20 mg.), testosterone (1 mg.), Kendall's ketone from oxidation of cortical hormone (1 mg.), scillaridin A (1 mg.), allocholesterol (5 mg.), epiallocholesterol (5 mg.), cholestenone (50 mg.), cholesterolene (10 mg.), cholesterol dicarboxylic acid (10 mg.), dihydrocholesterol (50 mg.), and epidi-hydrocholesterol (50 mg.), have all been found negative on rats. The dosages of each compound are given in parentheses. This result with scillaridin A is of special interest since the formula ascribed to this substance has double bonds between the 5-6 and 7-8 carbons in Ring B just as does ergosterol. In scillaridin A the hydroxyl group is attached to C_{14} instead of C_3 and the side chain is replaced by a ring.

DISCUSSION

The evidence seems convincing that the contaminant of spinal cord cholesterol which exhibits the four absorption bands is not ergosterol but 7-dehydrocholesterol, or some similar derivative with two double bonds in Ring B. We believe that provitamin D in heated, purified cholesterol is something quite different, which does not have these double bonds. Our experience indicates that

crude cholesterol also contains this provitamin D as a second contaminant, since we have been able to eliminate the four typical bands from the absorption spectrum of spinal cord cholesterol and still have a product so potent that 0.1 to 3.0 mg. equals 1 rat unit.

The difference in response of chicks and rats to ergosterol, 7-dehydrocholesterol, corn oil phytosterol, 22-dihydroergosterol, and scillaridin A is evidence that the two double bonds in Ring B are not alone responsible for antirachitic potency, but that the presence and configuration of side groups are important at least in influencing the degree of potency. Such variations in effectiveness might arise from differences in absorbability or specificity of action after these substances enter the blood stream.

SUMMARY

1. Absorption spectra studies show that the provitamin D of heated, purified cholesterol is not 7-dehydrocholesterol or any compound showing the four absorption bands.

2. The chick assay demonstrates that the contaminant of spinal cord cholesterol which does exhibit the four bands is not ergosterol, but 7-dehydrocholesterol or some similar compound.

3. The more favorable chick response to irradiated corn oil phytosterol indicates that the contaminant of this sterol is not ergosterol.

4. In preparing 7-dehydrocholesterol by the method described by Windaus *et al.*, we have obtained a product analyzing as $C_{27}H_{44}O_2$, but with a potency similar to Windaus' 7-dehydrocholesterol and two other products having antirachitic potency but differing in melting points and specific rotation.

We wish to express our thanks to Dr. David Klein of The Wilson Laboratories for cholesterol used in the preparation of 7-dehydrocholesterol, to The Grisard Laboratories for a sample of scillaridin A, to Dr. E. C. Kendall for the cortical hormone preparation, and to Dr. T. F. Gallagher for samples of cholesterol dicarboxylic acid, dihydrocholesterol, epidihydrocholesterol, and cholesterolene. We wish also to recognize the careful work of Miss Betty Stoerkel in feeding the experimental animals and preparing the line test.

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THE WATER AND ELECTROLYTE DISTRIBUTION AMONG PLASMA, RED BLOOD CELLS, AND MUSCLE AFTER ADRENALECTOMY*

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In recent years many investigators have studied the changes in the serum electrolyte pattern produced by adrenalectomy and Addison's disease. Little attention seems to have been paid, however, to the effects which these changes may have upon the water and electrolyte contents of the cells which are bathed by the plasma and which are in some sort of equilibrium with it. Red blood cells and muscle appear at once to be favorable material for studies of this sort, both because of their physiological importance and because of ease of handling. It is with the changes taking place in these tissues in "adrenal insufficiency" that the present paper is chiefly concerned.

Method

Cats were used as experimental animals throughout. Approximately 15 cc. of blood were drawn from the normal animal by heart puncture, and as soon thereafter as possible the soleus muscle was aseptically removed from one leg, a local anesthetic being used to avoid possible water and electrolyte disturbances resulting from general anesthesia. After a sufficient time to allow the animal to recover from the effects of this operation the adrenals were removed in two stages, at least 10 days intervening between the two operations.¹ Following the removal of the second adrenal gland the animal was maintained in a normal state for several days by means of cortin injections to obviate possible

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complications due to operative shock. The cortin was then withheld and the animal kept under observation during the next several days until signs of severe or moderately severe adrenal insufficiency were evident (lack of appetite, muscular weakness, etc.). At this time a second blood sample was taken and the soleus muscle from the other leg was removed. The animal was then sacrificed.

The technique of handling blood and muscle was the same in all cases. Heparin was used as the anticoagulant throughout. A small sample of blood was taken for hematocrit measurement. The centrifuge used for this purpose revolved at 14,000 R.P.M., and at this speed 20 minutes sufficed to insure constant cell volumes. Determinations were made in duplicate and repeated if the first two failed to agree within 1 per cent. The remaining blood was centrifuged at 3000 R.P.M. for 45 minutes, the plasma drawn off, and a 5 cc. sample measured into a tared platinum crucible. Another small sample served for freezing-point measurement (Johlin (1)). The plasma and white cells remaining above the erythrocytes were removed with a medicine dropper, and the red cells poured into a second tared crucible. After thorough mixing a hematocrit measurement was made on the packed cells to determine the amount of plasma remaining among them. This was never greater than 4 per cent. A sample of the packed cells was pipetted into a small tared flask of known volume (about 2.5 cc.) and weighed to determine the density. These cells were returned to the crucible. After weighing, the plasma and cells were dried to constant weight at 65° (MacLeod (2)) to determine the per cent of water. Finally the dried material was ashed in a muffle furnace at 600° for 20 hours, and the ash carefully washed into 10 cc. volumetric flasks and made up to volume. The chemical analyses were made on suitable aliquots.

The muscle upon removal was wiped free of blood and divided approximately in half. One portion was used for chloride measurement, the open Carius method of Van Slyke (3) serving for this purpose. The other portion was placed in a platinum crucible, weighed, dried to constant weight, and ashed in the muffle furnace.

Potassium was measured by the colorimetric chloroplatinate method as described by Yoe (4). The method of Salit (5) was

used for Na measurements. Plasma chloride was measured by the adsorption indicator method described by Burlingame and Kirk (6). All analyses were carried out in duplicate or (when any doubt attached to the results) in triplicate. Contrary to the accepted belief we do not find a loss of Cl from the plasma of cats in the dry ashing process. The results so obtained have been compared with analyses made on samples of unashed plasma, with the adsorption indicator method of Saifer and Kornblum (7). The latter method gives the lower results by 3 or 4 per cent.

TABLE I
Plasma Electrolytes in Experimental Adrenal Insufficiency

Experiment No.	M.-eq. per kilo water									Per cent water			Freezing point	
	Sodium			Potassium			Chloride							
	Before	After	Per cent difference	Before	After	Per cent difference	Before	After	Per cent difference	Before	After	* Per cent difference	Before	After
													°C.	°C.
1	188	147	-22.4	57.2	+60	147	135	-12	91.1	92.8	+1.87	-0.640	-0.640	
2	177	129	-26.4	49.9	+127	142	118	-17	91.9	90.2	-1.85	-0.630	-0.640	
3	180	142	-21.3	95.6	+44	141	123	-13	91.0	91.8	+0.88	-0.655	-0.645	
4	181	150	-17.3	54.6	+31	144	129	-10	89.8	90.5	+0.78	-0.650	-0.620	
5	175	138	-21.3	96.5	+67	136	121	-18	91.5	91.0	-0.55	-0.625	-0.640	
6	175	143	-18.3	74.6	+24	138	119	-14	91.2	91.0	-0.22	-0.645	-0.575	
7	172	134	-22.3	56.2	+77	140	110	-21	91.1	89.5	-1.76	-0.645	-0.645	
8	171	124	-27.4	03.0	-25	140	113	-19	91.7	91.5	-0.22			

In the case of the cells the figures resulting from the analyses are corrected for the amount of substance in the plasma remaining among them.

Results

Reference to Table I shows that the plasma electrolyte changes which we measure are in the same direction and of the same magnitude as those found by other investigators both in cats and in other animals. There is a marked loss of both Na and Cl and an even more marked (in per cent of initial amount) increase in K. It is to be noted that the Na loss considerably exceeds that of Cl, a fact first commented upon by Baumann and Kurland (8). Moreover, although we have not made HCO_3 analyses, we may assume

from the work of others that its concentration is also diminished (Swingle and Eisenman (9); Harrop *et al.* (10)). This would also be suggested by the decreased Na:Cl ratio in adrenal insufficiency.

The effects of the observed ion losses on the freezing point are apparently compensated by an increase in undetermined crystalloids, for we have observed no regular change in freezing point (Table I). It seems likely that urea (which is known to increase several fold after adrenalectomy) plays some part. However, the maximum urea increases observed by other investigators (Harrop *et al.* (11)) are not great enough to maintain the freezing point at the level observed in all but one experiment. Ca, Mg, PO_4 , and other substances are also known to increase in the plasma of "insufficient" animals, but the initial concentrations of these substances are so small that even a 100 per cent increase in all of them would have only a slight effect on the freezing point. Whatever the substances may be which are responsible for the maintenance of the osmotic pressure of total plasma they are largely such as can penetrate red cells freely, for the modified plasma is hypotonic with respect to the *normal* cells, as indicated by the fact that they take up water.

After consideration of the electrolyte changes which occur in the plasma in adrenal insufficiency it is obvious that the red cells must undergo considerable change in order to remain in osmotic equilibrium with their new environment. This change may be (1) a loss of osmotically active material, (2) an uptake of water, or (3) a combination of (1) and (2). This combination is, in fact, what occurs. The uptake of water is shown by the dry weight measurements. The loss of ions by diffusion is shown by calculating the concentrations of the several ions in the "insufficient" cells, not in terms of the water actually present, but as concentrations which would be found had no water entered.² The data

² The figures for per cent difference of Na and K (Table II) are obtained by calculating ion concentrations in normal cells per unit of both water (a) and dry substance (b). Those of the "insufficient" cells are calculated per unit of dry substance (c); the per cent difference between (b) and (c) is subtracted from or added to (a) to give a figure of concentration in the cell water which would have obtained if equilibrium had been reached only by loss of electrolytes. Expressing cell concentrations in terms of water facilitates comparison with plasma concentrations.

in Table II for per cent difference of sodium and potassium are in the corrected form, so that the differences between these and the figures found for the normal controls represent the true loss or gain of ions across the red cell membrane.

It is to be noted that alterations in plasma electrolyte concentration are roughly paralleled by altered cell concentrations. This is true even of the cations Na and K to which the red cell envelope is commonly supposed to be impermeable. As further evidence for the cation exchange across the red cell membrane it may be pointed out that the cells never swell to the extent which is predicted by a calculation based on the plasma cation

TABLE II
Electrolytes in Red Blood Cells in Experimental Adrenal Insufficiency

Experiment No.	M.-eq. per kilo water						Per cent water			Hematocrit per cent cells		
	Sodium			Potassium								
	Before	After	Per cent difference	Before	After	Per cent difference	Before	After	Per cent difference	Before	After	Per cent difference
1	170	148	-22	5.6	8.1	+46	66.0	69.2	+4.85	38.7	25.0	-35
2	163	126	-23	5.4	11.1	+106	65.2	66.1	+1.38	35.7	47.9	+34
3	159	138	-18	5.0	7.0	+40	65.0	65.5	+0.77	38.8	44.4	+14
4	167	153	-14	5.5	6.2	+13	64.5	65.5	+1.55	34.9	35.1	
5	157	149	-5	5.1	6.7	+31	64.6	67.0	+3.71	37.9	47.9	+26
6	159	135	-15	4.9	11.4	+133	63.7	67.4	+5.80	38.6	49.0	+26
7	157	142	-10	4.8	5.7	+19	64.5	65.5	+1.55	36.8	43.1	+19
8	166	149	-10	4.2	7.5	+79	64.7	65.8	+1.70	40.2	39.2	

loss and the assumption that the cells are perfect osmometers; i.e., neither lose nor gain cations. In a typical experiment (No. 5), if we assumed the cells to be perfect osmometers containing the observed amount of water initially, it may be predicted from the new plasma Na and K concentrations that they will swell from an initial volume of 100 to a new volume of 113. The observed volume as calculated from the measured water uptake is only 103. If, however, the Na and K loss and gain by the cells are taken into account in the first calculation, the predicated volume would be 107. The smaller difference between this and the observed volume is at least in part accounted for on the basis of the Ca, Mg,

and other cation increases which are known to occur, but which we have not measured.

Muscle—The muscles, like the red blood cells, might be expected to change in composition in order to remain in equilibrium with the altered plasma. Several investigators (Silvette and Britton (12); Winter and Hartman (13)) have found that the muscles of "insufficient" cats and rats respectively contain on the average several per cent more water than those of normal controls. This is not invariably the case in cats (Table III), for the differences in water content in most cases are no greater than those which one finds between matched muscles of normal animals. In any event the interpretation of changes in muscle water is complicated by the

TABLE III
Muscle Electrolytes in Experimental Adrenal Insufficiency

Experiment No.	K per kilo intracellular H ₂ O, m.-eq.			Chloride interspace, gm. H ₂ O per 100 gm. muscle			Per cent water		
	Before	After	Per cent difference	Before	After	Per cent difference	Before	After	Per cent difference
1	149	156	+4.7	15.6	14.4	-7.7	76.4	77.4	+1.3
2	150	160	+6.7	16.3	16.4		76.6	76.5	
3	144	146	+1.4	15.6	16.7	+7.3	76.7	76.6	
4	145	160	+10.3	17.4	14.1	-19.0	74.9	74.8	
5	150	150		15.3	14.1	-7.7	74.3	78.1	+5.1
6	147	145	-1.4	15.6	16.5	+5.8	75.3	77.3	+2.7
7	148	162	+9.5	16.7	17.8	+6.6	76.4	76.7	

fact that all the water found upon drying is not intracellular, an appreciable portion of it coming from capillaries and intercellular spaces. Moreover, these two portions need not necessarily be affected in the same degree, or even in the same direction by given plasma variations. We have therefore attempted to differentiate between the two fractions by following the accepted assumption that *all* muscle Cl is in the intercellular space, and that its concentration here is the same as it is in the plasma. The values for interspace calculated in this way (Table III) agree well with those first calculated by Overton (14) and subsequently by Fenn *et al.* (15) on frog muscle. We feel, however, that if any consistent difference exists between the volume of the interspaces in

normal and adrenalectomized cats, it is of such a magnitude as to be within the error of the Cl method used in these experiments, which admittedly is rather great in our hands. If, however, the intercellular volume is the same (as is indicated in these measurements), then any change in water content of the whole muscle must be due to the intracellular portion. In three of the seven experiments recorded there is a significant increase in water content. Such an increase is to be expected as a result of the hypotonicity of the plasma after adrenalectomy. The absence of such a gain in most of the experiments could result if the muscles lost osmotically active material. Analyses were made for potassium, which is the principal cation of muscle, but reference to Table III reveals that no loss in this ion occurs. In fact, the tendency seems to be rather toward an increased concentration. Thus in Experiments 1, 2, 4, and 7 the increase is definite. Of this group only Experiment 1 shows a simultaneous increase in water content, which means that the magnitude of the K increase per unit of dry weight is greater than that recorded. In Experiments 5 and 6, in which the water uptake is greatest, there is no increased K concentration, but again, if calculated per unit of dry weight, an inward migration of K will be revealed. Assuming K to be the principal osmotically active substance in muscle, the evidence tends toward the view that the tonicity of the intracellular fluid has increased in spite of the decreased tonicity of the extracellular fluid. Whatever the significance of this phenomenon may be, it is obvious that muscle does not serve as the source of the high plasma K in adrenal insufficiency.

DISCUSSION

The idea of the exchange of cations across the red blood cell membrane is not new, but at the present time, owing largely to the work of Doisy and Eaton (16), Wakeman, Eisenman, and Peters (17), and Van Slyke, Wu, and McLean (18), it is almost universally believed that it does not occur, at least within the normal range of plasma variation. Kerr (19), however, has shown that experimental procedures which alter the cation concentration of dog serum may also alter the red cell concentration in such a way as to indicate that the cells are permeable to these ions. This has recently been substantiated by Yannet, Darrow,

and Cary (20). The latter authors, however, also find that under the same conditions the red cells of rabbits and monkeys (in which K is the principal cation) are impermeable.

The present data indicate conclusively, we believe, that under conditions in which the electrolyte balance of cat plasma is markedly upset a redistribution of cations may take place across the red cell membrane.

To this interpretation two possible objections may be raised: (1) that the red cells in adrenal insufficiency are not normal, their permeability having been altered by a lack of the cortical hormone, and (2) that the cells in the second blood sample are new cells formed in the interim between removal of the second adrenal and withdrawal of the blood sample, entering the blood stream with the new observed Na and K contents.

Opposed to both objections is the evidence presented in the following paper (21) that changes of the same sort and quantitatively of a similar order may occur in 24 hours in the cells of cats whose plasma Na levels have been reduced by intraperitoneal injections of isotonic glucose. In these animals there can be no question of a lack of cortical hormone, and the duration of the experiment is such as to preclude the possibility of new cell formation to account for the observed results.

It has been suggested by Gilman (22) that the increase in per cent volume of blood cells, which is regularly observed in the blood of adrenalectomized animals, is entirely or largely due to migration of water from the modified plasma into the cells and tissues, and that this water shift accounts adequately for the diminished plasma volume. Our data do not confirm this assumption. In the five experiments in which the hematocrit did increase the combined H_2O uptake of red cells and muscle is inadequate to account for the observed changes.

SUMMARY

From a study of the water and electrolyte distribution in adrenalectomized cats the following conclusions are drawn.

Blood Changes

1. The plasma osmotic pressure as measured by the freezing point remains practically unchanged after adrenalectomy, although both the Na and Cl levels are greatly reduced.

2. The loss of Na and Cl from the plasma renders it hypotonic to the red cells, as evidenced by the fact that they take up water.

3. Simultaneously with the water uptake the total cation content of the cells diminishes in relation to dry weight. The diminished cation content results from the outward migration of Na.

4. The rise in the plasma K concentration is accompanied by an increased cell concentration. The K increase in the red cells, however, does not compensate for the Na decrease.

5. From (3) and (4) it follows that under the conditions in the adrenalectomized cat the red cells are cation-permeable.

Muscle Changes

6. The total water content of muscle may increase or remain constant. The increase when present is intracellular.

7. In either case the K content of the muscle per unit of dry weight is increased.

8. The latter finding indicates conclusively that the high plasma K is not due to K liberation from muscle.

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THE WATER AND ELECTROLYTE DISTRIBUTION BETWEEN PLASMA AND RED BLOOD CELLS FOLLOWING INTRAPERITONEAL INJECTIONS OF ISOTONIC GLUCOSE*

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In a previous paper (1) we have shown that the alterations in the cation concentrations of the plasma which result from adrenal insufficiency in the cat, are accompanied by similar changes in their concentrations in the red blood cells. These changes persist even after correction is made for the slight water shift to the cells, and we have concluded that a change in the plasma electrolyte pattern may so affect the red cell membrane as to make it somewhat permeable to cations. As has been pointed out in the previous paper, two serious objections may be raised to our interpretation of these results: (1) the altered permeability is not due to the electrolyte change alone, but partly to a lack of cortical hormone which may play a part in the maintenance of normal permeability; (2) there has been no cation exchange at all, the cation differences which we have measured being due to the presence of new red cells of different cation contents which entered the circulation in the interim between the drawing of the two blood samples. These objections disappear if it can be shown that the cell cation concentrations still change when the plasma electrolytes are altered rapidly in otherwise normal animals. It is with the removal of these objections that the present paper is concerned.

It has been shown by several authors (Gilman (2); Darrow and Yannet (3)) that large intraperitoneal injections of isotonic glucose solution will markedly alter the plasma electrolyte picture. Furthermore, the changes produced are very similar to those

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found in adrenal insufficiency; indeed, the procedure has been used by several workers in the field of adrenal physiology to simulate this condition (Silvette and Britton (4); Parkins, Taylor, and Swingle (5)). Since, moreover, the changes can be brought about fairly rapidly by this means, it seemed admirably suited to our purpose.

While these experiments were in progress, a paper by Yannet, Darrow, and Cary (6) appeared in which these authors, in experiments like our own, were able to show that, although the red blood cells of the dog may leak cations, those of the rabbit do not. Since no adequate explanation of this difference was offered, we decided to extend our experiments to include the rabbit, and these results are reported here along with those on cats.

EXPERIMENTAL

Considerable experimentation with the volume of glucose injected and the length of time which it was allowed to remain in the peritoneal cavity led to a standard procedure which has been followed in all of the experiments reported in this paper. A 15 cc. sample of normal blood was drawn by heart puncture and 65 cc. of isotonic (5.5 per cent) glucose per kilo of body weight were injected intraperitoneally. This was followed in 4 hours by a paracentesis and the reinjection of another quantity of isotonic glucose solution equal in volume to the ascitic fluid withdrawn. After a second 4 hour period another paracentesis was performed, and as much fluid as possible withdrawn. The total amount of fluid withdrawn was always very nearly equal to the amount injected. About 16 hours later a second blood sample was drawn by heart puncture and the animal killed. Thus a period of approximately 24 hours elapsed between the drawing of the two blood samples.

The methods of handling the blood and of analysis were the same as in the previous paper, except that K was determined by Hubbard's (7) modification of the Kramer and Tisdall cobalt-nitrite method, and plasma Cl by the adsorption indicator method of Saifer and Kornblum (8).

Results

Inspection of Table I will show that the changes in plasma electrolytes induced by this procedure are very like those resulting

from adrenal insufficiency. There is a fall in the concentrations of Na and Cl but generally an increase in that of K. One differ-

TABLE I

Cat Plasma Electrolytes before and after Intraperitoneal Glucose Injections

Experiment No.	M.-eq. per kilo water									Per cent water		
	Sodium			Potassium			Chloride			Before	After	Per cent difference
	Before	After	Per cent difference	Before	After	Per cent difference	Before	After	Per cent difference			
1	175	163	-6.9	3.7	5.5	+48.7				91.8	90.2	-1.7
2	169	151	-10.7	3.7	5.0	+35.0	140	119	-15.0	90.5	88.3	-2.4
3	164	129	-21.4	3.9	4.7	+20.5				90.7	87.6	-3.4
4	181	148	-18.2	4.4	4.8	+8.9	147	122	-17.0	89.3	90.3	+1.1
5	171	146	-14.6	4.4	3.9	-11.4				91.5	90.2	-1.4
6	175	136	-22.3	4.1	4.5	+9.8	145	115	-20.6	90.4	89.6	-0.9
7	144	129	-10.4	2.8	4.9	+75.0	130	111	-14.5	91.2	90.2	-1.1
8	168	145	-13.7	3.6	4.4	+22.2	140	122	-12.7	91.5	90.5	-1.1
9	171	137	-19.3	6.2	4.6	-25.8	146	116	-20.3	90.4	88.0	-2.7
10	148	137	-7.4	3.0	2.9	-3.3	136	120	-11.8	89.3	87.5	-2.0

TABLE II

Electrolytes in Cat Red Blood Cells before and after Intraperitoneal Glucose Injections

Experiment No.	M.-eq. per kilo water						Per cent water		
	Sodium			Potassium			Before	After	Per cent difference
	Before	After	Per cent difference	Before	After	Per cent difference			
1	169	166	-1.8	4.9	4.9	0.0	64.6	64.7	+0.15
2	163	157	-3.7	5.0	5.7	+14.0	65.3	65.5	+0.31
3	168	155	-7.7	5.3	5.0	-6.0	65.3	65.0	-0.46
4	170	150	-11.8	5.1	5.6	+9.8	65.5	65.3	-0.31
5	165	154	-6.7	4.9	5.5	+12.2	64.9	65.5	+0.93
6	163	155	-4.9	5.9	6.1	+3.4	65.5	66.0	+0.77
7	149	146	-2.0	4.0	6.1	+52.5	65.0	65.9	+1.39
8	152	148	-2.6	5.0	5.8	+16.0	64.7	65.8	+1.70
9	157	146	-7.0	8.4	12.1	+46.3	65.9	68.3	+3.64
10	157	154	-1.9	5.2	6.5	+25.0	64.5	65.7	+1.86

ence which warrants mention is that in the glucose experiments the losses of Na and Cl are more nearly equal than in the adrenal-ectomized animals, where the loss of Na greatly exceeds that of Cl.

Table II shows the changes in the Na and K contents of the red blood cells which accompany the plasma changes. As in the adrenalectomized cats, the cell Na falls when the plasma Na falls, and the cell K changes with the plasma K, although not always in the same direction. The changes in the water content of the cells in these experiments are less marked than in the adrenalectomized

TABLE III

Electrolyte Content of Rabbit Plasma before and after Intraperitoneal Glucose Injections and of Red Blood Cells Following Intraperitoneal Glucose

Experiment No.	M.-eq. per kilo water									Per cent water		
	Sodium			Potassium			Chloride					
	Before	After	Per cent difference	Before	After	Per cent difference	Before	After	Per cent difference	Before	After	Per cent difference
Plasma before and after glucose injections												
1	148	142	-4.0	4.8	4.0	-16.5	116	109	-6.0	92.8	92.8	
2	146	134	-8.2	5.7	4.6	-19.4	115	106	-7.8	93.4	92.0	-1.50
3	148	136	-8.1	5.2	9.9	+93.4	116	103	-11.2	93.1	92.0	-1.18
4	149	129	-13.4	4.3	9.9	+130.0	117	85	-27.4	91.8	89.4	-2.62
5	152	126	-17.1	3.6	5.8	+62.3	119	92	-22.7	91.7	89.8	-2.07
6	147	130	-11.6	2.9	3.6	+25.5	116	103	-11.2	92.6	91.3	-1.40
7	151	136	-10.0	5.6	4.7	-17.0	120	94.5	-21.2	91.3	90.5	-0.88
Red blood cells following glucose injections												
1				166.0	162.3	-2.2				67.0	67.2	+0.30
2				148.0	130.0	-12.2				68.5	67.8	-1.02
3				172.5	167.2	-3.4				67.4	69.2	+2.70
4				149.5	137.4	-8.1	76.5	63.0	-17.7	66.5	65.0	-2.30
5				150.5	136.0	-9.7	85.2	57.1	-33.0	66.2	66.7	+0.76
6				153.0	140.4	-8.2				66.5	66.0	-0.75
7				154.0	148.3	-3.7				66.0	67.8	+2.72

animals; indeed in several cases there is none. Where a change did occur, however, it was corrected for in calculating the Na and K concentrations in the cells of the second blood sample. Thus the differences in the cell Na and K before and after the glucose injections (Table II) really represent a migration of ions across the red cell membrane and not merely a decrease (in the case of Na) in concentration due to the uptake of water.

It will be noted that the percentage changes in cell Na are much smaller than in the case of K. Indeed, several of them do not exceed the errors attached to the analyses. However, the regularity with which the decrease occurs and the fact that in at least half of the experiments it greatly exceeds the experimental error lead us to believe that it is real.

In most of the experiments the ascitic fluid, as well as the blood, was analyzed for Na, K, and Cl and the results of the analyses show that the plasma and fluid were approximately in equilibrium with respect to these ions. Since the total volume of the ascitic fluid withdrawn was always considerably greater than the estimated plasma volume, it follows that the total amount of Na, K, and Cl removed was always greater than the amount of them initially present in the circulating plasma. In spite of this the plasma K rose in most cases. The chief source of these excess ions is undoubtedly the interstitial fluid, but some of them probably came from cells. The particular cells involved we do not know. Thaller has recently shown that in cats, in which the K concentration of the plasma has been raised by hemorrhage, the muscle K does not fall, indicating that leakage from this tissue is not responsible for the rise in plasma K observed. In a few experiments we have analyzed muscle as well as blood and our results confirm Thaller (9). In view of the work of D'Silva (10) it seems likely that the liver is the source of the K.

The results of the experiments upon rabbits are summed up in Table III. The Na and Cl of the plasma fall, and to about the same extent, but the plasma K may show either a rise or a fall. The cell K, however, always shows an unmistakable fall. We are at loss for an explanation of why our results differ from those of Yannet, Darrow, and Cary. Unfortunately their paper does not contain sufficient data to make a detailed comparison possible.

DISCUSSION

The results of these experiments show, we believe, that when the electrolyte balance of the plasma is altered, the red blood cells of cats and rabbits, at least, become somewhat permeable to cations. The question of the cortical hormone as a possible factor in affecting their permeability does not enter, since the adrenal glands of all of these animals were intact. Furthermore

the duration of the experiments was so short as to preclude the entrance into the circulation of a sufficient number of red cells of changed Na and K concentration to affect the results.

The particular cation predominating in the cells appears to have no influence upon this permeability change. In the red cells of the cat the principal base is Na, as is also the case in dogs. Rabbit red blood cells, on the other hand, resemble those of the

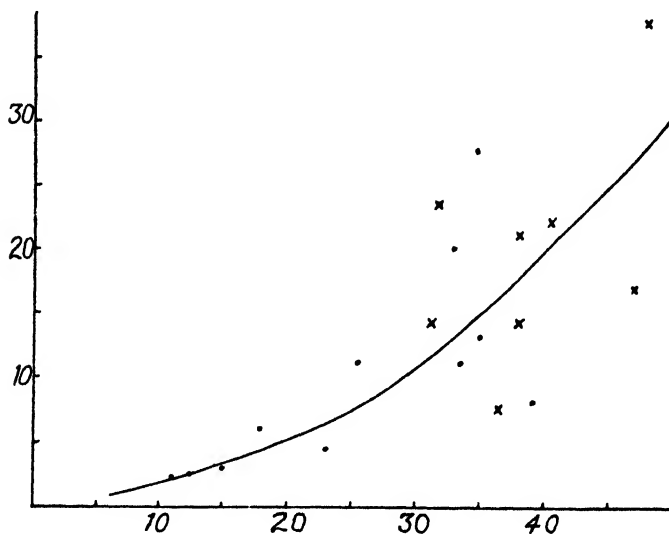


FIG. 1. The relation between the Na loss from the plasma and the Na loss from the red blood cells. The abscissæ represent plasma Na loss in milliequivalents per kilo of plasma water and the ordinates cell Na loss in milliequivalents per kilo of cell water. The crosses indicate the losses in adrenalectomized cats and the dots the losses in cats which received intra-peritoneal glucose injections.

monkey and of man in having K as the predominant base. This difference in red cell base is given by Yannet, Darrow, and Cary as the explanation for the dissimilarities which they observed between dogs on the one hand and rabbits and monkeys on the other. As has been pointed out above, we do not confirm their results in rabbits.

To emphasize further the similarity between the blood changes in adrenal insufficiency and in the present glucose experiments we

have plotted the Na data from both series of experiments on the same graph (Fig. 1). It will be noted that the cell Na losses increase as the plasma Na losses increase. The relation is obviously not a linear one, and we have arbitrarily drawn a curve which seems to fit the points best. No such relation can be demonstrated between cell K loss and plasma K loss, but this is not surprising in view of the rapidity with which the plasma K can change.

There is one other point in connection with these experiments which we believe warrants mention. Silvette and Britton have shown that, by using intraperitoneal glucose injections, they could reduce the Na and Cl concentrations of cat plasma below the level commonly found in adrenal insufficiency without killing their animals. This has not been our experience. A comparison of Table I of this paper with Table I of the preceding one shows that, in general, the fall in plasma Na and Cl in our glucose experiments was less than in the adrenalectomized animals. In several of the preliminary experiments large single injections of glucose were made instead of two smaller ones and many of these resulted in the death of the animals before the second blood sample was drawn. Other animals, which survived, showed the same symptoms as cats in an advanced stage of insufficiency; *i.e.*, lack of appetite, disinclination to move about, and a swaying gait when forced to walk. Moreover, the animals used in the experiments reported in this paper also showed these symptoms, although usually to a less marked degree.

SUMMARY

1. The plasma of cats and rabbits which have received large intraperitoneal injections of isotonic glucose shows changes in its electrolyte content which are very like those found in adrenal insufficiency. These are a fall in the concentrations of Na and Cl but generally a rise in that of K.

2. With the changes in the concentrations of the cations of the plasma there are always changes in their concentration in the cells, even after correction is made for any water shift which may occur. This leads us to conclude that when the electrolyte balance of the plasma is sufficiently altered, the red blood cell membrane may become somewhat permeable to cations.

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A STUDY OF KETOSIS IN PRIMATES

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There have been numerous investigations on the production of acetone by the mammalian organism (1-9). In the diabetic or fasting animal most investigators have assumed that no carbohydrate was oxidized, and that the energy arose from the oxidation of fat and protein. The latter could be calculated from the urinary nitrogen, and the remainder of the calories were taken to be due to fat oxidation. In the experiments on Primates (1-4) the complete oxidation of 2 moles of fatty acids required the simultaneous oxidation of 1 mole of antiketogenic material; and each additional mole of fatty acid oxidized gave rise to 1 mole of acetone substances. These results could not be confirmed in experiments on the rabbit, goat, pig, dog, or rat (4-8). The authors found that only a mild ketonuria developed after prolonged fasting, and they concluded that the fatty acids were oxidized without the simultaneous oxidation of antiketogenic materials. However, no determinations were made of the actual metabolic mixture in these experiments.

In previous experiments on the white rat (9) there was an attempt to make the most accurate determinations of the food-stuffs oxidized and to calculate the amounts of acetone that should arise from such metabolic mixtures according to Shaffer's theory. The calculated acetone production was found to be in fair agreement with the amounts recovered in the urine, and the discrepancies could be attributed to errors inherent in the experimental technique. In the present experiments similar data were collected on Primates.

Method

Eight observations were obtained on seven monkeys of various species. The animals were fasted and injected with 100 mg. of

phlorhizin per kilo daily. The foodstuffs metabolized were estimated from analyses of the metabolic rate, R.Q., urinary nitrogen, and acetone substances. The metabolic rate and R.Q. were determined with a closed respiratory, indirect calorimeter for

TABLE I
Ketosis in Primates

Experiment No.	Species and sex	Weight	Length of experiment	Oxygen	R.Q.	Nitrogen	Glucose	Theoretical yields of acetone	Acetone recovered	Change in R.Q. to make (9) equal (10)
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
		<i>kg.</i>	<i>hrs.</i>	<i>liters</i>		<i>mg.</i>	<i>mg.</i>	<i>mm</i>	<i>mm</i>	
1	Macaque, ♀	3.2	23	42.5	0.708	1592	3502	43.5	44.1	-0.001
2	" ♀	3.0	21	39.4	0.709	1025	1342	49.7	50.9	-0.002
3	Green, ♂	4.1	20	33.7	0.702	694	1045	47.6	20.0	+0.015
4	Macaque, ♂	3.1	26	43.0	0.685	1330	3541	81.3	43.7	+0.023
5	Baboon, ♀	7.2	13	37.0	0.724	2010	970	11.2	2.2	+0.004
6			19	62.1	0.709	2120	1546	61.9	12.2	+0.016
7	Green, ♂	3.0	16	29.3	0.715	570	960	23.0	1.4	+0.015
8	" ♀	2.8	38	28.5	0.702	1440	2364	37.7	2.7	+0.020

TABLE II
Metabolic Study of One Human (Grafe (10))

Date	Oxygen	R.Q.	Nitrogen	Theoretical yields of acetone	Acetone recovered	Change in R.Q. to make (6) equal (5)
(1)	(2)	(3)	(4)	(5)	(6)	(7)
	<i>liters</i>		<i>mg.</i>	<i>mm</i>	<i>mm</i>	
Oct. 8	351.5	0.715	7430	212	37	0.011
" 12	287.3	0.704	5730	371	123	0.007
" 13	298.8	0.702	5290	408	167	0.010
" 14	331.5	0.712	4920	301	119	0.019
" 15	301.1	0.704	4860	397	157	0.027
" 16	298.1	0.700	4530	447	132	0.027

periods which varied from 13 to 38 hours. The urine excreted during the same periods was collected and aliquot portions were analyzed for nitrogen, acetone substances (10), and sugar. Assuming a ketogenic-antiketogenic ratio of 2:1, the theoretical

yields of acetone were calculated and compared with the amounts recovered. The method of calculation of the theoretical yields and the factors used were fully described and discussed by Shaffer (2).

Results

The results are presented in Table I. It may be seen that the theoretical yields exceeded the amounts in the urine six times, and were less on two occasions. In our previous studies on the white rat (9) it was found that such discrepancies could be accounted for by errors in the method of determining the R.Q. We have therefore calculated the change in the R.Q. required to account for the differences between the theoretical and actual yields of acetone. These changes (Column 11) varied from -0.002 to $+0.023$, averaging $+0.012$.

A search of the literature revealed one group of observations by Grafe (11) on a catatonic human patient which could be used for the calculation of the ketogenic-antiketogenic balance. In these experiments the patient received no food, the metabolism was determined for periods of 2 hours, and a 24 hour urine specimen was analyzed. In view of the fact that the patient was immobile throughout the experiment, it is probable that the 2 hour metabolism determination was a fair measure of the metabolic rate for the entire 24 hour period. The calculation of these data is presented in Table II. It may be seen that the discrepancies between the theoretical and actual yields of acetone could be accounted for by changes of the R.Q. which varied from 0.007 to 0.027, with an average of 0.017.

DISCUSSION

The calculation of the quantitative acetone excretion according to Shaffer's theory is based on the knowledge of the amounts of the primary food substances oxidized. It is therefore evident that the accuracy of these calculations is dependent on the estimation of the foodstuffs burned. Other investigators have used two general methods for the analysis of the metabolic mixture. The subjects were examined under conditions in which the carbohydrate oxidized could be considered negligible; namely, prolonged fasting or diabetes. It was assumed that the metabolic energy

arose from the oxidation of fat and protein. In an earlier paper (9) we were able to show that prolonged fasting in rats could not exhaust the carbohydrate stores of the body. If we assumed that no carbohydrate was oxidized in these experiments, a considerable error resulted in the subsequent calculation of the quantitative acetone metabolism. In view of the work of Houssay (11) on the relation of pancreas and the pituitary, the assumption that no carbohydrate is oxidized by the diabetic animal may be questioned. The second method by which the problem was approached was the determination of the metabolic rate and the R.Q. for short periods, while the urinary constituents were determined on a 24 hour specimen. It is obvious that these results would be invalidated by variations which occur in the metabolic rate during the 24 hours. On the other hand, the collection of urine specimens for short periods introduces an error, since the excretion of the end-products of metabolism may lag far behind the oxidation of the food substances.

In the present experiments an effort was made to circumvent these sources of error by determining the total metabolism for periods of 24 hours, and collecting the urine voided during the same period. Two sources of error remained which we were unable to correct; namely, the excretion of acetone through the lungs, and the errors of the methods used. In six of the present eight experiments the theoretical yields of acetone exceeded the amounts recovered in the urine. A correction for the pulmonary excretion of acetone would therefore tend to reduce the discrepancies found. We have calculated the changes required in the experimental data to account for the differences between the theoretical and actual recoveries of acetone substances and compared them with the errors of the methods. It was found that the changes of the R.Q. were significant. The accuracy of the methods was estimated from a series of alcohol checks performed throughout the course of these experiments. The maximum deviation from the theoretical alcohol check was 0.010, and the average deviation was 0.006. In the present report the changes of the quotient required to account for the discrepancies were within 3 times the average experimental error in six of the eight experiments. It therefore seems highly probable that in Primates, as in the white rat, the complete oxidation of ketogenic

substances requires the simultaneous oxidation of a definite proportion of antiketogenic foodstuffs.

SUMMARY

In eight experiments on seven phlorhizinized monkeys the foodstuffs metabolized have been determined from the oxygen consumption, the R.Q., and the urinary nitrogen. Assuming a ketogenic-antiketogenic ratio of 2:1, the amounts of acetone that should be excreted have been calculated and compared with the amounts recovered in the urine. A close agreement was observed. The discrepancies could be accounted for by errors inherent in the methods of analysis. It therefore seems highly probable that the complete oxidation of ketogenic substances requires the simultaneous oxidation of a definite proportion of antiketogenic foodstuffs.

The author takes great pleasure in acknowledging the aid of Professors John F. Fulton and Harold E. Himwich.

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SYNTHESIS OF SERINE

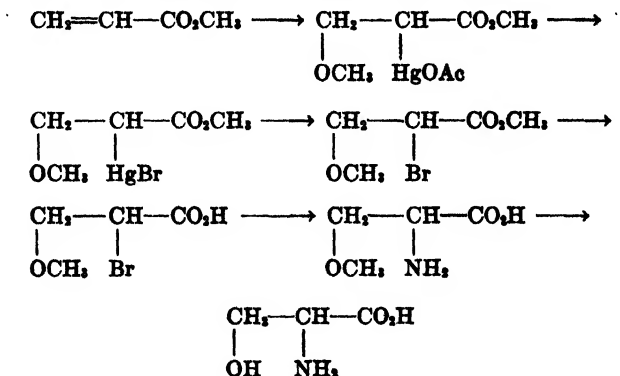
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(Received for publication, September 17, 1936)

The difficulties encountered in the synthesis of aminohydroxy acids are well illustrated in the case of serine. Although several different methods of preparing serine have been proposed, the only practical one is that reported recently by Dunn, Redemann, and Smith (1). It therefore seemed desirable to determine the feasibility of synthesizing serine by the mercuration method of Schrauth and Geller (2). Methyl acrylate, the starting material, is now a readily available chemical,¹ so that the method appeared to be an excellent one, if a satisfactory yield of serine could be obtained.

The reactions involved are shown in the following equations.



Each of the steps has been studied under various conditions. Certain difficulties were encountered, but these have been satis-

¹ We are indebted to the Röhm and Haas Company for supplying us with the solution of methyl acrylate in methyl alcohol used in this investigation.

factorily worked out so that yields of serine ranging from 30 to 40 per cent are obtained by this method.

There are a few points which should be mentioned. Most of the trouble was met with in the preparation of the bromomercuri ester, since that substance is unstable and somewhat soluble in aqueous methyl alcohol. To avoid these difficulties the mercuriation was carried out in a small volume of methyl alcohol and was not allowed to proceed longer than 3 days or at temperatures above 35°. The bromomercuri ester has not been obtained in a crystalline state and hence cannot be purified. It is essential, therefore, that the chloroform solution of the crude ester be carefully washed and dried to remove methyl alcohol and water. Otherwise the bromination does not proceed smoothly.

To obtain the best yields in the hydrolysis of the bromo ester it is necessary to use dilute alkali and to keep the temperature below 40°. 2 N sodium hydroxide gave poor results. α -Amino- β -methoxypropionic acid is much more soluble than serine in water and aqueous alcohol. Better yields are obtained, therefore, if no attempt is made to purify the aminomethoxy acid. The demethylation with hydrobromic acid is best carried out on small amounts of material. After removal of the hydrobromic acid it is convenient to combine several of the residues for recrystallization.

EXPERIMENTAL

Methyl α -Bromo- β -Methoxypropionate—150 gm. of a 60 per cent solution of methyl acrylate in methyl alcohol were placed in a 1 liter flask. 60 gm. of methyl alcohol and 320 gm. of mercuric acetate were added. The mixture was allowed to stand at room temperature for 3 days with occasional shaking. A slight excess of the methyl acrylate solution was used to allow for deterioration of the reagent, since the pure methyl acrylate was not isolated. At the end of 3 days the flask was cooled in an ice bath and a solution of 120 gm. of potassium bromide in 400 cc. of water was added during 10 minutes. The bromomercuri ester precipitated as a heavy oil which was extracted with 800 cc. of chloroform. The aqueous layer was extracted with 200 cc. of chloroform. The combined extracts were washed three times with water and dried over anhydrous sodium sulfate. The chloroform solution was

filtered and placed in a 3 liter flask exposed to direct sunlight. 150 gm. of bromine were added slowly, a large excess being avoided. The flask was shaken frequently and the temperature of the reaction mixture was held below 45° by occasional cooling. 30 to 45 minutes were required for the addition of the bromine. The solution was then cooled in an ice bath and the mercuric bromide was filtered. The chloroform was removed under reduced pressure through a 20 cm. column and the residue was distilled. After a small amount of low boiling material was removed, the rest was collected in one fraction boiling from 70–80° at 6 mm. The crude bromo ester weighed 160 to 170 gm. (81 to 86 per cent of the theoretical yield) and was used in the next step without further purification. Fractionation of the crude material through a Widmer column yielded pure methyl α -bromo- β -methoxypropionate.

$C_5H_9O_3Br$. Calculated, Br 40.61; found, Br 40.70

In addition to the pure methyl α -bromo- β -methoxypropionate there was obtained a small amount of higher boiling material whose bromine content was much higher. The isolation of α -bromoacrylic acid from the hydrolysis of this fraction indicated that it contained methyl α, β -dibromopropionate.

The bromination can be carried out in the absence of sunlight, if a strong electric light is used and the reaction is allowed to proceed at a higher temperature. However, more time is required and a poorer yield is obtained.

α -Bromo- β -Methoxypropionic Acid—125 gm. of crude bromo ester were shaken with 1800 cc. of 0.5 N sodium hydroxide in a 3 liter bottle. The ester dissolved completely in 5 minutes. 450 cc. of 2 N sulfuric acid were slowly added. The solution was concentrated to 500 cc. under reduced pressure and then extracted with six 300 cc. portions of ether. The ether extracts were combined, washed once with a small volume of cold water, and dried. The ether was distilled, leaving crude α -bromo- β -methoxypropionic acid. The yield was 105 to 110 gm. (90 to 95 per cent of the theoretical amount). This material was used in the next step. Fractionation of the crude bromo acid yielded pure α -bromo- β -methoxypropionic acid boiling at 91° at 2 mm. The distillation was accompanied by some decomposition which be-

came quite appreciable if a large amount of the acid was distilled at one time.

$C_4H_7O_3Br$.	Calculated.	Br 43.72, neutral equivalent 183
	Found.	" 43.64, " " 181

α -Amino- β -Methoxypropionic Acid—50 gm. of the crude bromo acid were heated with 500 cc. of concentrated ammonium hydroxide for 4 hours at 80–90°. Ordinary 500 cc. glass bottles can be used for this reaction, if they are heated slowly and the temperature is not allowed to exceed 85°. The solution was concentrated to dryness under reduced pressure, water was added, and the solution was reconcentrated to dryness. The residue was allowed to stand under 500 cc. of acetone until the gummy mass had solidified to a light brown solid. The solid was filtered, dried, and used in the next step without purification. Pure α -amino- β -methoxypropionic acid was obtained by dissolving the amination mixture in the least possible volume of water and adding 6 volumes of absolute alcohol. On cooling overnight in the ice box, the amino acid precipitated. Two recrystallizations from aqueous alcohol gave a pure white product melting with decomposition at 200–210°.

$C_4H_7O_3N$.	Calculated.	C 40.34, H 7.56, N 11.76
	Found.	" 40.27, " 7.42, " 11.84

The benzoyl derivative was prepared in the usual manner. After one recrystallization from water it melted sharply at 147–148°.

$C_{11}H_{13}O_4N$.	Calculated.	N 6.28, neutral equivalent 223
	Found.	" 6.17, " " 221

The formyl derivative of α -amino- β -methoxypropionic acid was prepared by the method of Fruton and Clarke (3). It melted at 151–152° after recrystallization from absolute ethyl alcohol.

$C_5H_7O_4N$.	Calculated.	N 9.52, neutral equivalent 147
	Found.	" 9.43, " " 146

Serine—55 gm. of the crude amination mixture were dissolved in 300 cc. of constant boiling hydrobromic acid and the solution was refluxed for 3 hours. The hydrobromic acid was removed

under reduced pressure. Water was added and the solution was again concentrated to dryness. The residue was dissolved in water and concentrated ammonium hydroxide was slowly added until the odor of ammonia persisted after shaking the solution. The excess ammonium hydroxide was removed by concentration to dryness. The residue was dissolved in 200 cc. of hot water and the solution was heated with norit for 5 minutes and filtered. 2 to 3 volumes of alcohol were added and the mixture was cooled overnight in the ice box. The product obtained contained a small amount of ammonium bromide. Recrystallization from 70 per cent alcohol yielded 12 to 15 gm. of pure serine (31 to 39 per cent over-all yield).

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